

OXIDATIVE STRESS IN VERTEBRATES AND INVERTEBRATES

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Molecular Aspects of Cell Signaling

Edited by

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“Live as if you were to die tomorrow. Learn as if you were to live forever.”

Mohandas Karamchand Gandhi

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PREFACE

All oxygen-utilizing animals and organisms have to deal with reactive oxygen species (ROS), which include superoxide anions, hydroxyl, alkoxyl, and peroxy radicals, and hydrogen peroxide. These radicals are common products of life in an aerobic environment, and they are responsible for oxygen toxicity. Proteins, lipids, and nucleic acid are targets for ROS attack, and modification of these molecules can increase the risk of chronic neurodegenerative diseases, visceral diseases, and cancer. “Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling” provides readers with a comprehensive description of the latest research on oxidative stress and antioxidant defenses in vertebrate and invertebrate systems. In biological systems, cells respond to mild oxidative stress by inducing antioxidant defenses and other protective systems. The antioxidant capacities of tissues are well matched to the rates of oxygen consumption and radical production. In vertebrate and invertebrate systems a variety of endogenous antioxidants (reduced glutathione) and antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase) act in a concerted manner to protect tissues against oxidative damage. The balance among oxidants and antioxidant enzyme systems, levels of antioxidants, and endogenous antioxidant mechanisms may be of major importance in the protection against oxidative stress-mediated cell injury. Under normal conditions, the rate of production of oxidants is balanced by the rate of oxidant elimination. However, an imbalance between prooxidants and antioxidants results in oxidative stress. Increase in ROS production has a substantial impact cellular metabolism and may lead to either defective cellular function and aging or chronic neurodegenerative and visceral diseases. Therefore, a

better understanding of the roles of ROS-mediated signaling in normal cellular function as well as in disease is necessary for the development of therapeutic agents for oxidative stress-related chronic diseases.

Unlike other edited books that focus on oxidative stress in mammals, this unique book provides a comparative account of oxidative stress and antioxidant defenses in vertebrates and invertebrates, dealing not only with basic mechanisms and biomarkers but also with oxidative stress-mediated chronic diseases. This edited book is a valuable source of information for both basic scientists and clinicians who are interested in basic mechanism and oxidative stress-associated diseases. In this book, chapters are organized into two sections: (1) oxidative stress in vertebrates (Chapters 1–17) and (2) oxidative stress in invertebrates (Chapters 18–26), followed by a perspective (Chapter 27).

In Part I, Chapters 1 and 2 deal with the generation of ROS in the brain and their signaling associated with neural cell survival, cell suicide, and diseases. Chapters 3 and 4 discuss mitochondrial DNA mutation-induced oxidative stress underlying biochemical and pathological consequences and redox therapy in mitochondrial diseases and changes in kainic acid-induced neurotoxicity, which can be implicated in the pathogenesis of neurotraumatic and neurodegenerative diseases. Chapter 5 covers the historical aspects of the discovery of NF-E2-related factor 2 (Nrf2), recent advances in molecular aspects of its function, and updates involving Nrf2 association with various pathological conditions. Chapter 6 discusses modulation of oxidative stress by caloric restriction, suggesting that a calorie-restricted diet and the composition of diet may significantly improve ROS homeostasis both in single cells as well as in the whole body. Chapters 7–10

deal with the contribution of oxidative stress and inflammation to the pathogenesis of neurodegenerative diseases (Alzheimer disease, Parkinson disease). Chapter 11 summarizes free radical contribution to the development of cardiovascular diseases and discusses the applicability of antioxidant therapy based on data from clinical trials. Chapter 12 provides a comparison between vertebrates and invertebrates with regard to oxidative stress and aging. Chapter 13 addresses various environmental stressor-induced toxicities in experimental animals like rats and humans to elucidate the molecular mechanisms underlying oxidative stress. Chapter 14 discusses the role of selenoproteins in cellular redox regulation and signaling. Chapter 15 gives a clinical demonstration of the effectiveness of antioxidant administration in different diseases. Chapter 16 demonstrates that grape-derived bioactive polyphenolic components from wine effectively protect against the onset and progression of Alzheimer disease phenotypes, suggesting that moderate wine consumption may have preventive and/or therapeutic value in Alzheimer disease. Finally, Chapter 17 discusses pharmacological and therapeutic properties of propolis, a resinous mixture that honeybees collect from tree buds, sap flow, and other botanical sources, which is very beneficial for human health because of its richness in phenolic compounds.

In Part II, Chapter 18 reviews the endocrine control of oxidative stress in insects. Chapter 19 focuses on oxidative stress and innate immune system in airway epithelial cells of the fruit fly *Drosophila melanogaster*. Chapter 20 explores the molecular mechanisms of antioxidant protective processes in the honeybee *Apis mellifera*. Chapter 21 describes a hypothetical mechanism

associated with iron-induced oxidative stress, implicating ROS production in olfactory dysfunction in the honeybee brain. Chapter 22 covers cutting-edge information on the Keap1/Nrf2 system in flies as well as its implications in combating human diseases. Chapter 23 is devoted to orchestration of oxidative stress responses in *Drosophila melanogaster* and promoter analysis study of circadian regulatory motifs. Chapter 24 deals with the protective role of sestrins (a unique family of proteins that is critically involved in cellular defense) against chronic target of rapamycin complex activation and oxidative stress in *Drosophila*. Chapter 25 explores current advances in the studies of oxidative stress and age-related memory impairment in the nematode *C. elegans*. Chapter 26 elegantly reviews oxidative challenge and redox sensing in mollusks by focusing on effects of natural and anthropic stressors. Finally, Chapter 27 provides readers with an in-depth perspective on current progress on our understanding of oxidative stress. It also presents readers and researchers with information that will be important for future research dealing with oxidative stress.

Biochemists, neuropharmacologists, toxicologists, and clinicians will find this book useful for understanding basic mechanisms of oxidative stress in vertebrate and invertebrate systems. It is hoped that “Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling” will further stimulate young and senior scientists to perform research on oxidative stress and oxidative stress-associated diseases.

TAHIRA FAROOQUI
AKHLAQ A. FAROOQUI

FOREWORD

Oxidative stress is a cytotoxic process that occurs in cells when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS). This imbalance not only causes damage to important biomolecules (lipids, proteins, and nucleic acids) in cells, but also has an impact on functional activities in both vertebrates and invertebrates. This new volume entitled “Oxidative Stress in Vertebrates and Invertebrates: Molecular Aspects of Cell Signaling” brings together important information from expert researchers in the oxidative stress-mediated cell signaling area in both vertebrate and invertebrate systems. Accumulation of high levels of ROS and significant reduction in cellular redox systems are common processes associated with acute and chronic visceral and neurodegenerative diseases, including hypertension, preeclampsia, arteriosclerosis, acute renal failure, diabetes, and Alzheimer and Parkinson diseases. This well-organized book presents up-to-date and comprehensive information on oxidative stress-related signaling events in vertebrates and invertebrates. The text is clear, concise, and easily accessible. Subject matter is divided into a vertebrate section (17 chapters) and an invertebrate section (10 chapters). The editors are known for their work on oxidative stress and neurodegeneration. They have done a commendable job in putting together this volume, and have contributed 5 chapters. These editors have taken great care in selecting the topics and describing progress that has been recently made in this field. The authors of this book also tried to ensure uniformity and mode of presentation in a simple and clear manner.

Topics addressed in the vertebrate section include the generation of ROS and their roles in cell survival and

suicide; ROS-induced signal transduction and human diseases; biochemical and pathological consequences and redox therapy; pathogenesis of neurotraumatic and neurodegenerative diseases; oxidative stress mediated by caloric restriction; the role of oxidative stress and neuroinflammation in Alzheimer disease and Parkinson disease; selenoproteins in cellular redox regulation and signaling; antioxidant therapy and its effectiveness in oxidative stress-mediated disorders; pharmacological and therapeutic properties of propolis; comparison of oxidative stress in aging between vertebrates and invertebrates; and finally, oxidative stress-mediated signaling pathways by environmental stressors. The invertebrate section includes oxidative stress-induced signaling in three important phyla, namely, arthropoda, annelida, and mollusca. Topics addressed in this section include effect of oxidative stress on insect endocrine control; the innate immune system in airway epithelial cells of *Drosophila*; age-related memory impairment in *C. elegans*; olfactory learning and memory in *Apis mellifera*; Keap1/Nrf2 signaling in *Drosophila*; circadian rhythm in *Drosophila*; molecular antioxidant protective processes in *Apis mellifera*; protective role of sestrins against chronic TOR; and oxidative challenge and redox sensing in mollusks.

The subject matter in this book develops logically and progresses from one topic to another with an extensive bibliography along with major primary references. These references will help readers in pursuing their areas of interest. In order to facilitate better understanding and easier reading, this book also contains a large number of figures and line diagrams of signal transduction pathways. This book fills the gap between basic science and

clinical studies and provides the reader with the skills to apply basic science to clinical settings of chronic diseases associated with oxidative stress.

This book is essential reading material for a broad range of individuals, including researchers, clinicians, graduate and medical students, as well as the many health-conscious individuals who wish to know more about the emerging field of oxidative stress in vertebrate and invertebrate systems. It can be used as a supplemental text for a range of biology courses. It is anticipated that senior neuroscientists may also find

some inspiration from this book to overcome problems encountered in their research on oxidative stress in vertebrate and invertebrate systems.

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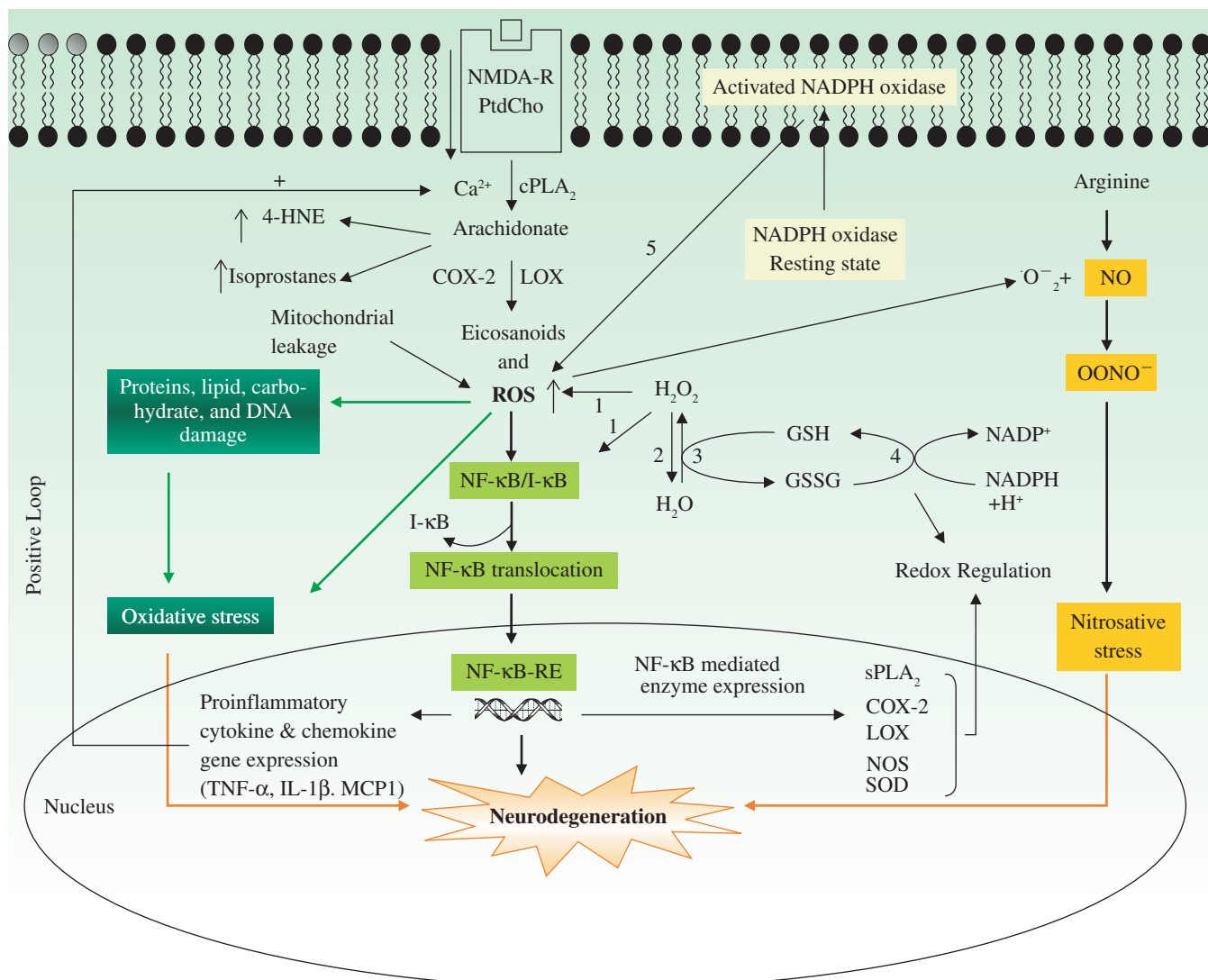


Fig. 1.2 Generation of reactive oxygen species (ROS) and enzymic and nonenzymic markers for oxidative stress. 1, Superoxide dismutase (SOD); 2, catalase; 3, glutathione peroxidase; 4, glutathione reductase; 5, NADPH oxidase. cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; 4-HNE, 4-hydroxynonenal; NO, nitric oxide; OONO⁻, peroxynitrite. Activation of NF-κB by ROS leads to its translocation to the nucleus, where it facilitates the transcription of proinflammatory enzymes (sPLA₂, COX-2, NOS, and SOD) and proinflammatory cytokines (TNF-α and IL-1β). These cytokines upregulate activities of cPLA₂ and sPLA₂ through a positive loop mechanism in cytoplasm and neural membranes. Upward arrows indicate increase in levels of metabolites.

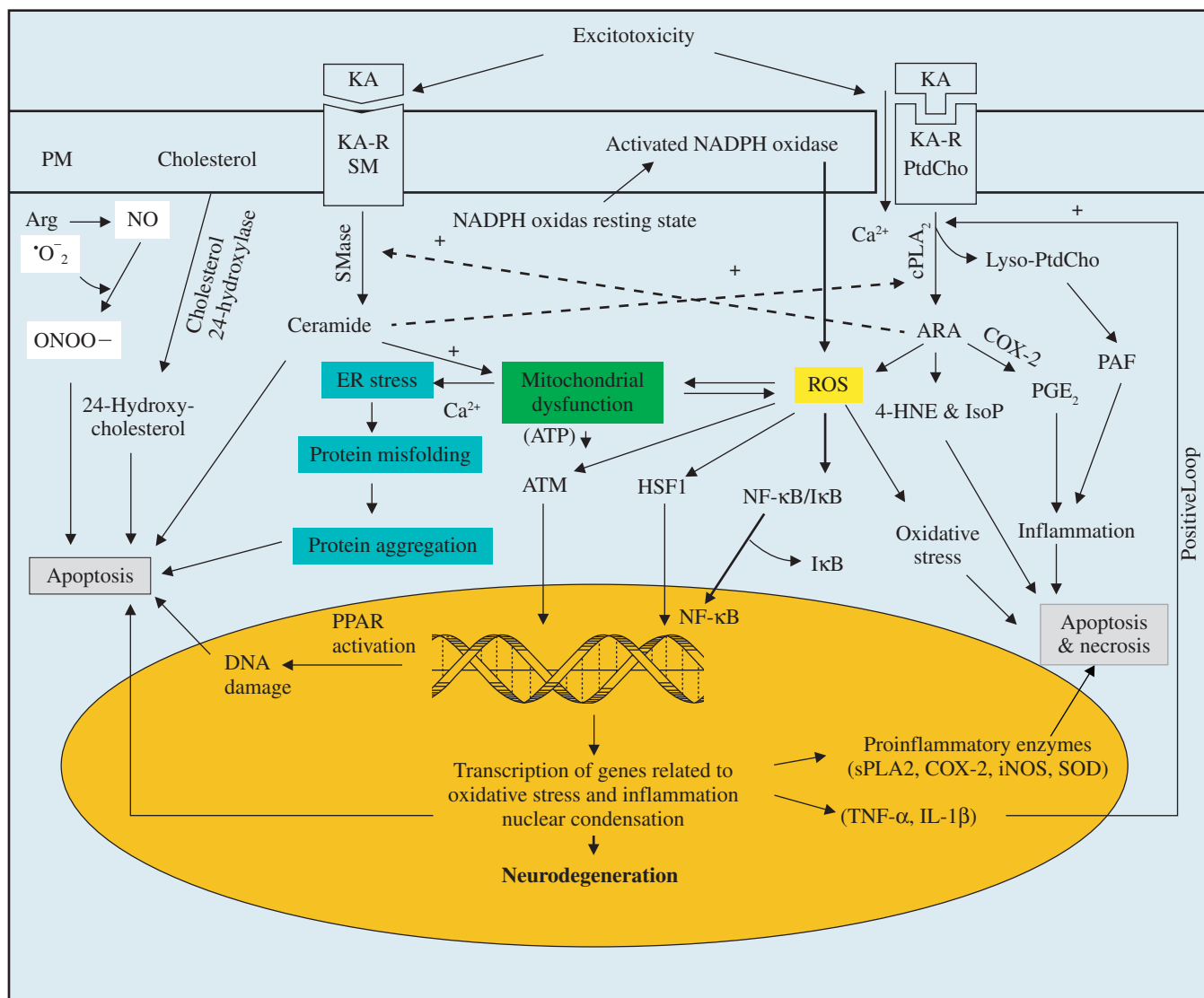


Fig. 4.4 Diagram showing interactions between glycerophospholipid and sphingolipid lipid-derived lipid mediators. KA, kainate; KA-R, kainate receptor; SM, sphingomyelin; SMase, sphingomyelinase; cPLA₂, cytosolic phospholipase A₂; PtdCho, phosphatidylcholine; ARA, arachidonic acid; Lyso-PtdCho, lyso-phosphatidylcholine; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; 4-HNE, 4-hydroxynonenal; PGE₂, prostaglandin E₂; PAF, platelet-activating factor; IsoP, isoprostane; Arg, L-arginine; NO, nitric oxide; ONOO⁻, peroxynitrite; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PARP, poly(ADP-ribose) polymerase; ATM, Ataxia-telangiectasia mutated; HSF-1, heat shock transcription factor 1. Positive sign (+) indicates stimulation.

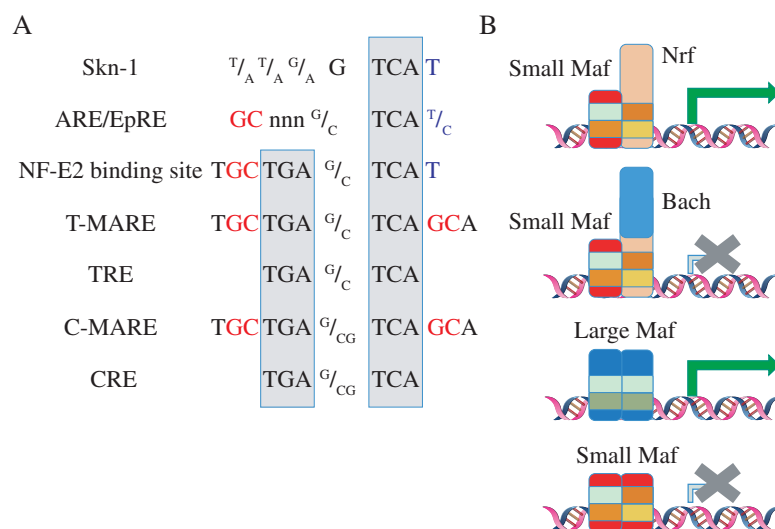


Fig. 5.2 DNA recognition sequences and the transcriptional activities of Maf-containing dimers. (A) Various MARE-related sequences are shown. The dinucleotide “GC” (marked in red) is essential for recognition by Maf proteins. The nucleotide “T” or “T/C” (marked in blue) enhances the binding of CNC proteins. Trinucleotides (boxed in gray) with the central G and GC sequence consist of the TRE and CRE, respectively, which make up the core region of the MARE. (B) Nrf1, Nrf2, Nrf3, and NF-E2 activate transcription by forming heterodimers with small Maf, while Bach1 and Bach2 repress transcription. A large Maf homodimer, possessing a *trans*-activation domain, activates transcription, while a small Maf homodimer, lacking a *trans*-activation domain, represses transcription. All of these Maf-containing dimers bind to T-MARE with high affinity.

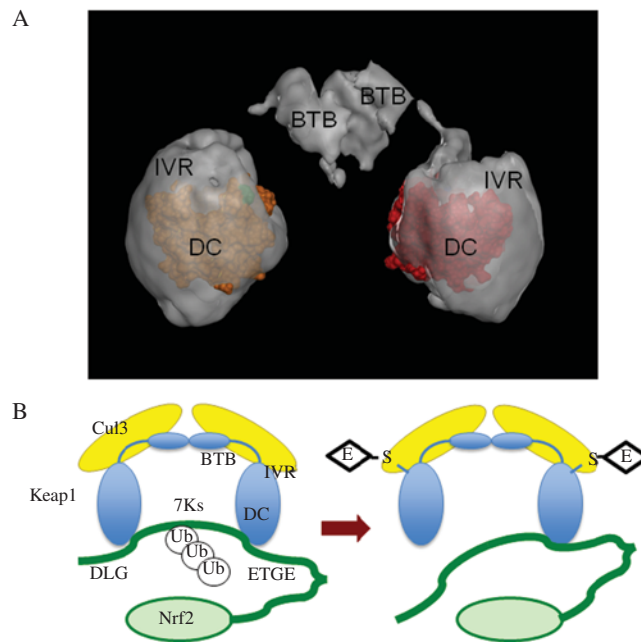


Fig. 5.6 The overall structure of the Keap1 homodimer and the regulation of Nrf2 activity by Keap1. (A) The three-dimensional structure of the Keap1 homodimer (cited from Ogura et al., *Proc Natl Acad Sci USA*. 2010:2842-7, 107). (B) Interaction between Nrf2 and the Keap1-Cul3 complex under unstressed conditions (left) and stressed conditions in which thiols are modified with electrophiles (E) (right). Each DC domain of the Keap1 homodimer binds to the DLG and ETGE motifs in the Neh2 domain of Nrf2. Keap1 is proposed to interact with Cul3 at the BTB domain and the IVR. Nrf2 is polyubiquitinated at 7 lysine residues (7Ks) between the DLG and ETGE motifs (left). Modification of Keap1 with electrophiles is thought to alter the overall conformation of the Keap1-Cul3 and Nrf2 complex, which inhibits Nrf2 ubiquitination (right).

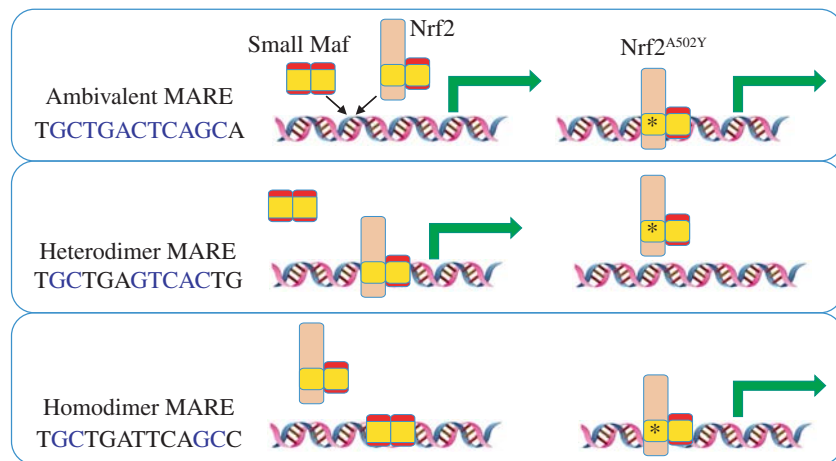


Fig. 5.8 Categorization of MARE-related sequences. Ambivalent MAREs are bound by both Nrf2-small Maf heterodimers and Maf homodimers. Heterodimer MAREs are preferentially bound by Nrf2-small Maf heterodimers, and homodimer MAREs are bound by Maf homodimers. Substitution of the alanine residue of the Nrf2 basic region with a tyrosine residue switches the DNA recognition specificity from the CNC type to the Maf type and results in the preferential binding of Nrf2 A502Y-small Maf heterodimers to homodimer MAREs and not to heterodimer MAREs.

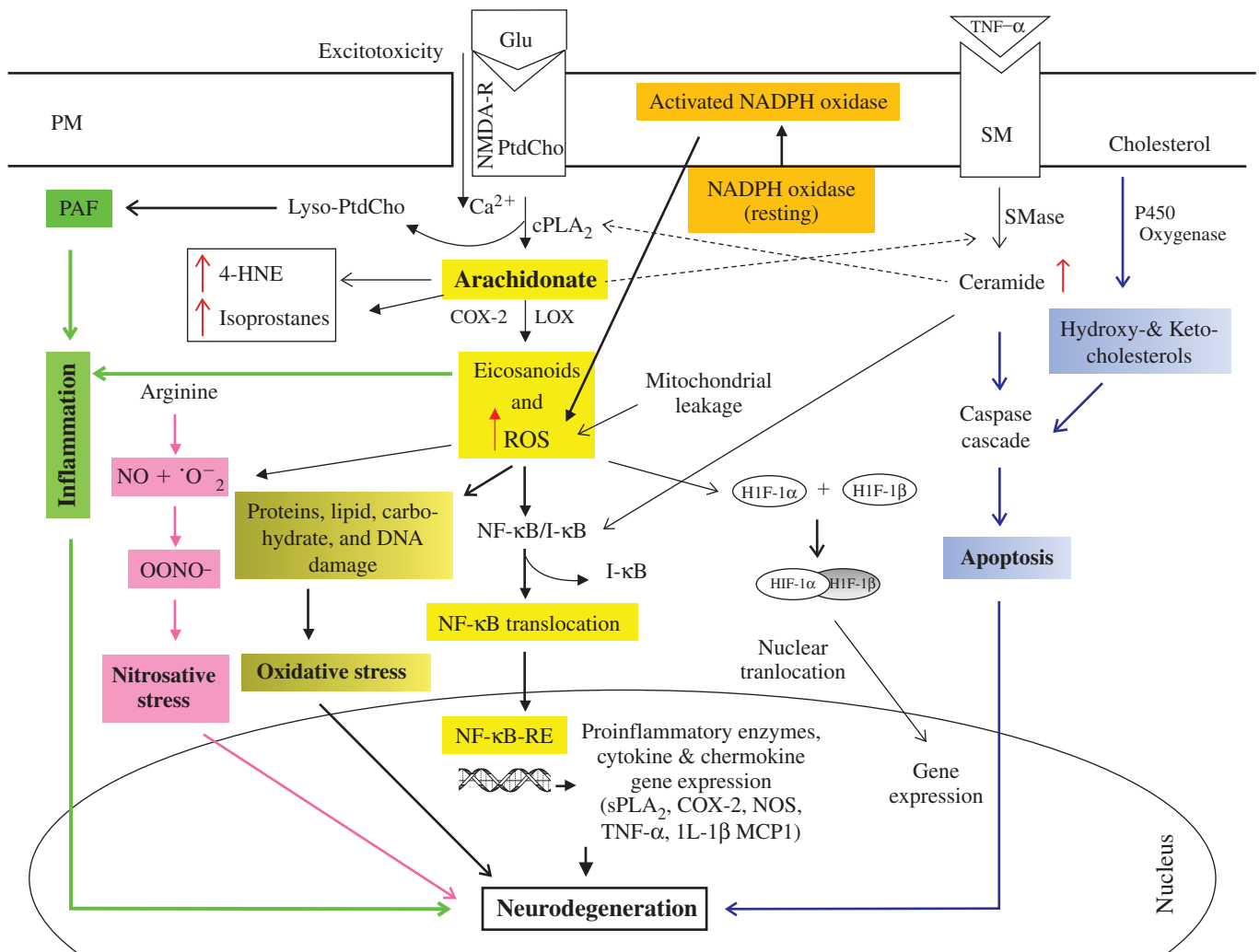


Fig. 7.3 Generation of lipid mediators and interactions between excitotoxicity, oxidative stress, and neuroinflammation in neurodegenerative diseases. cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; SMase, sphingomyelinase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin 1β; lyso-PtdCho, lyso-phosphatidylcholine; ROS, reactive oxygen species; HIF-1, hypoxia-inducible factor-1; 4-HNE, 4-hydroxynonenal; NO, nitric oxide; OONO⁻, peroxynitrite; PAF, platelet-activating factor. Activation of NF-κB by ROS leads to its translocation to the nucleus, where it facilitates the transcription of proinflammatory enzymes (sPLA₂, COX-2, NOS, and SOD) and proinflammatory cytokines (TNF-α and IL-1β). These cytokines upregulate activities of cPLA₂ and sPLA₂ through a positive loop mechanism in cytoplasm and neural membranes. Upward arrows indicate increase in levels of metabolites.

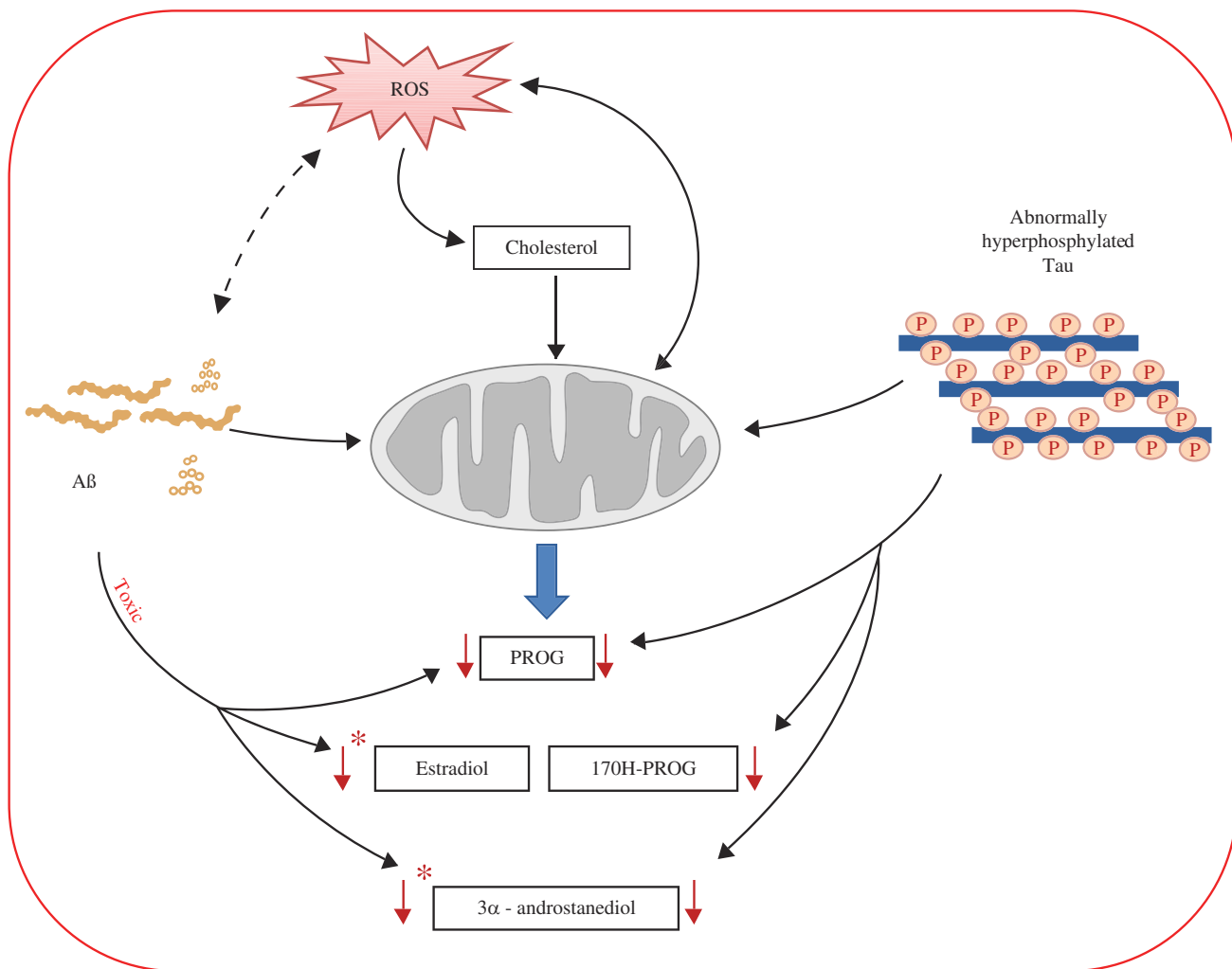


Fig. 8.3 Effect of toxic concentrations of Aβ peptides and abnormally hyperphosphorylated Tau protein on neurosteroid biosynthesis. Aβ induced a drop of the level of progesterone (PROG), estradiol, and 3α-androstanediol by acting on reactive oxygen species (ROS) formation and mitochondrial function and/or directly on steroidogenesis. The presence of abnormally hyperphosphorylated Tau protein had the same effect by inducing a decrease of progesterone, 17-hydroxyprogesterone (17OH-PROG), and 3α-androstanediol. On the other hand, it has been shown that nontoxic concentrations of Aβ induced an increase in estradiol and 3α-androstanediol levels (this pathway is marked by *).

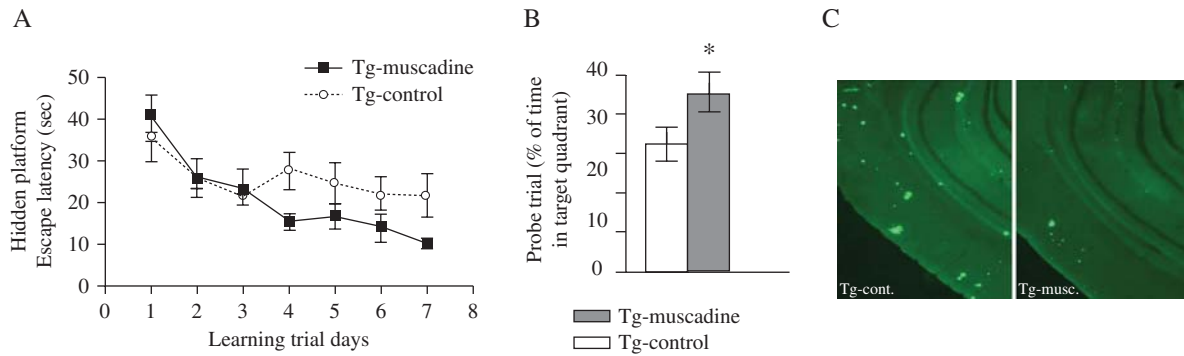


Fig. 16.4 Muscadine treatment improves spatial memory function and A β neuropathology in Tg2576 mice. (A,B) Assessments of spatial memory behavioral functions of 14 month old muscadine-treated (Tg-muscadine) and control, gender- and age-matched non-treated (Tg-control) Tg2576 mice using the Morris water maze protocol. (A) Learning trial hidden-platform acquisition curves. Tg-muscadine group performed significantly better than the control, non-treated group (Tg-control) [2-way ANOVA analysis of Tg-muscadine vs. Tg-control groups for muscadine treatment ($p < 0.05$, $F = 4.24$, $DFn = 1$, $DFd = 84$) and for training days ($p < 0.05$, $F = 6.43$, $DFn = 6$, $DFd = 84$)]. (B) Probe trial conducted 24 hours after completion of hidden-platform training. Muscadine-treated Tg2576 mice exhibited a significantly higher preference for the target platform compared to control, non-treated Tg2576 mice ($p < 0.05$, 2-tailed Student t test). In (A,B) Values represent group mean (+SEM); $n = 7-9$ mice per group. (C) Assessments of A β neuropathology reflected by amyloid neuritic plaque density in cerebral cortex and in the hippocampal formation of brain specimens from muscadine-treated and control, non-treated Tg2576 mice. Representative micrograph of brain specimen stained for amyloid neuritic plaques in muscadine-treated (Tg-musc.) or in control, non-treated (Tg-cont.) Tg2576 mice.

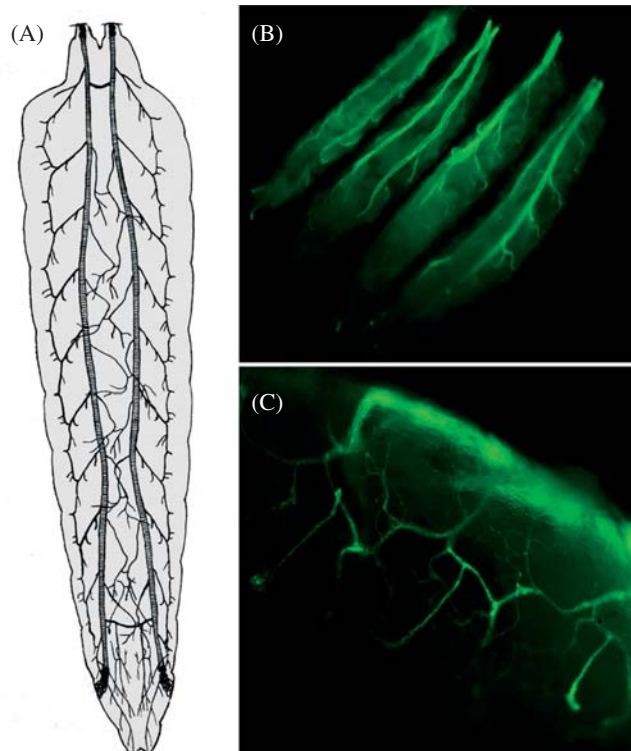


Fig. 19.2 Structure of the airway system of *Drosophila*. The airway system (trachea) of a larval fly is made up of interconnected tubes that deliver oxygen to almost every cell in the body (A). Upon stimulation with different stressors including infection, the airway epithelium launches a very effective response, comprising the expression of antimicrobial peptide genes (B and C, the latter at higher magnification).

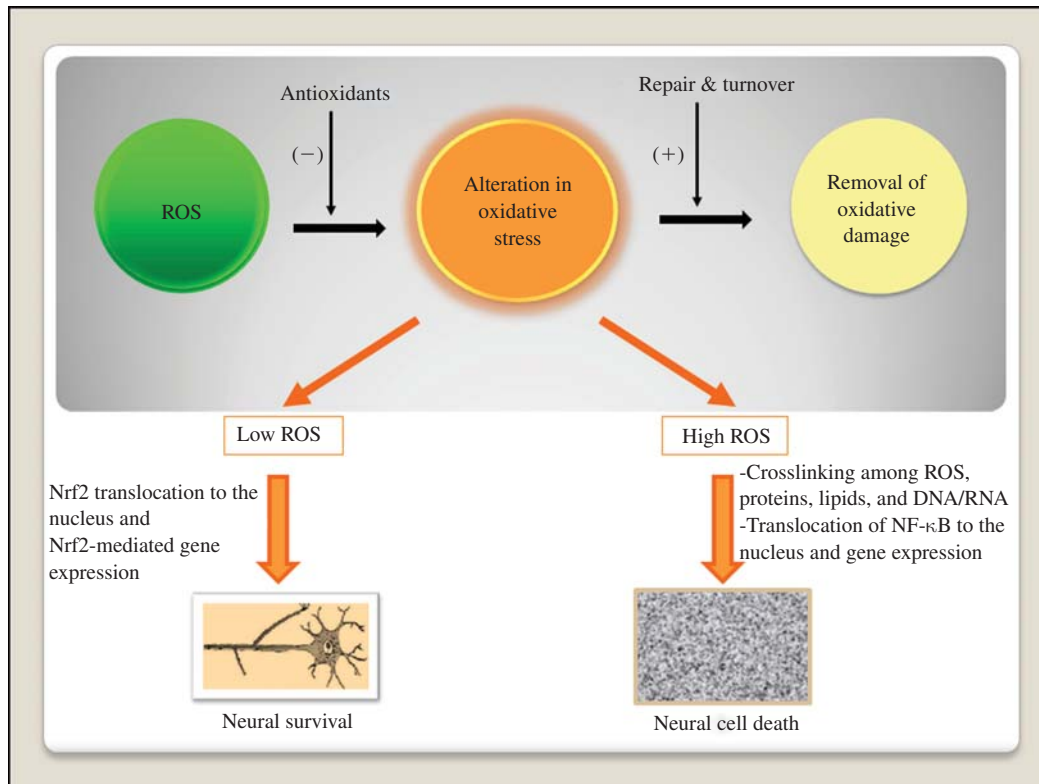


Fig. 21.2 Connection between reactive oxygen species (ROS)-mediated oxidative stress and abnormal functions. Increase in oxidant level, high ROS, decrease in antioxidants, and failure in repair and turnover result in functional abnormalities. Repair and turnover of oxidative damage, the so-called cellular defense mechanism, include DNA excision, resynthesis, and rejoining of DNA strands; repair of oxidized methionine residues in proteins; and normal membrane turnover releasing damaged lipids. Low ROS allow the translocation of nuclear factor-erythroid-2-related factor 2 (Nrf2) to the nucleus to regulate the expression of surviving genes for neural survival, whereas high ROS prevents translocation of Nrf2 to the nucleus. The cross-linking of high ROS occurs with proteins, lipids, DNA/RNA, which promotes neurodegeneration.

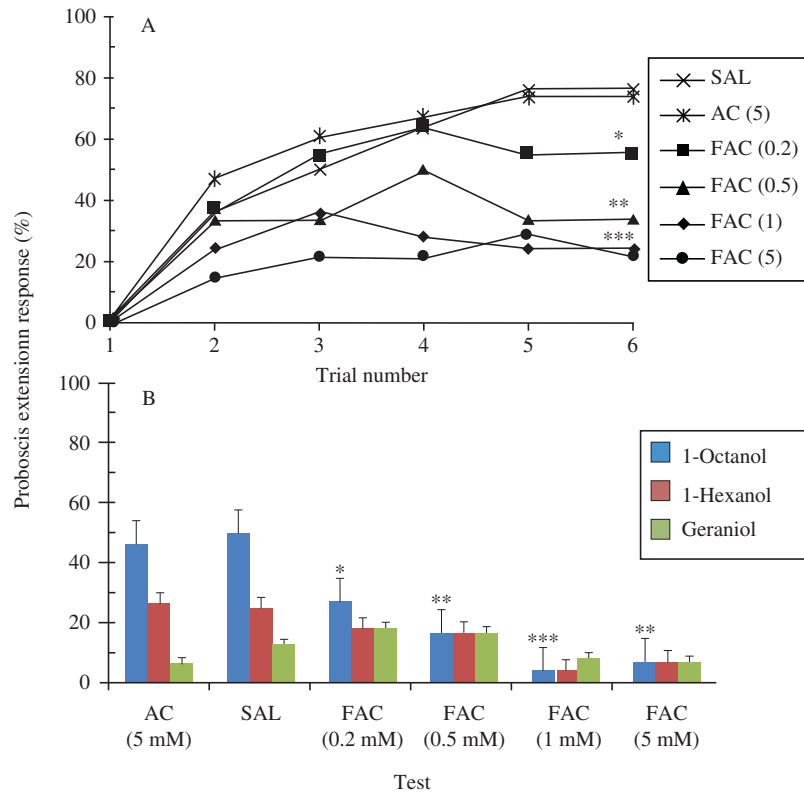


Fig. 21.3 Dose-response effect of ferrous ammonium citrate (FAC) on olfactory learning and memory. (A) Acquisition of the conditioned odor C: In this experiment, honeybees received 4 nl of saline (SAL), ammonium citrate (AC), or FAC at 0.2 mM, 0.5 mM, 1 mM, and 5 mM in each antennal lobe. Twenty-four hours later, subjects in each group were conditioned with 1-octanol (odor C). (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, molecularly similar (S), and molecularly dissimilar (D) odors in randomized order. *ns*, Not significantly different from controls (SAL, 5 mM AC). Asterisks indicate significant differences of respective points from control group: * $P = 0.05$, ** $P = 0.005$, 0.001, *** $P = 0.0001$). This figure is modified from reference [40].

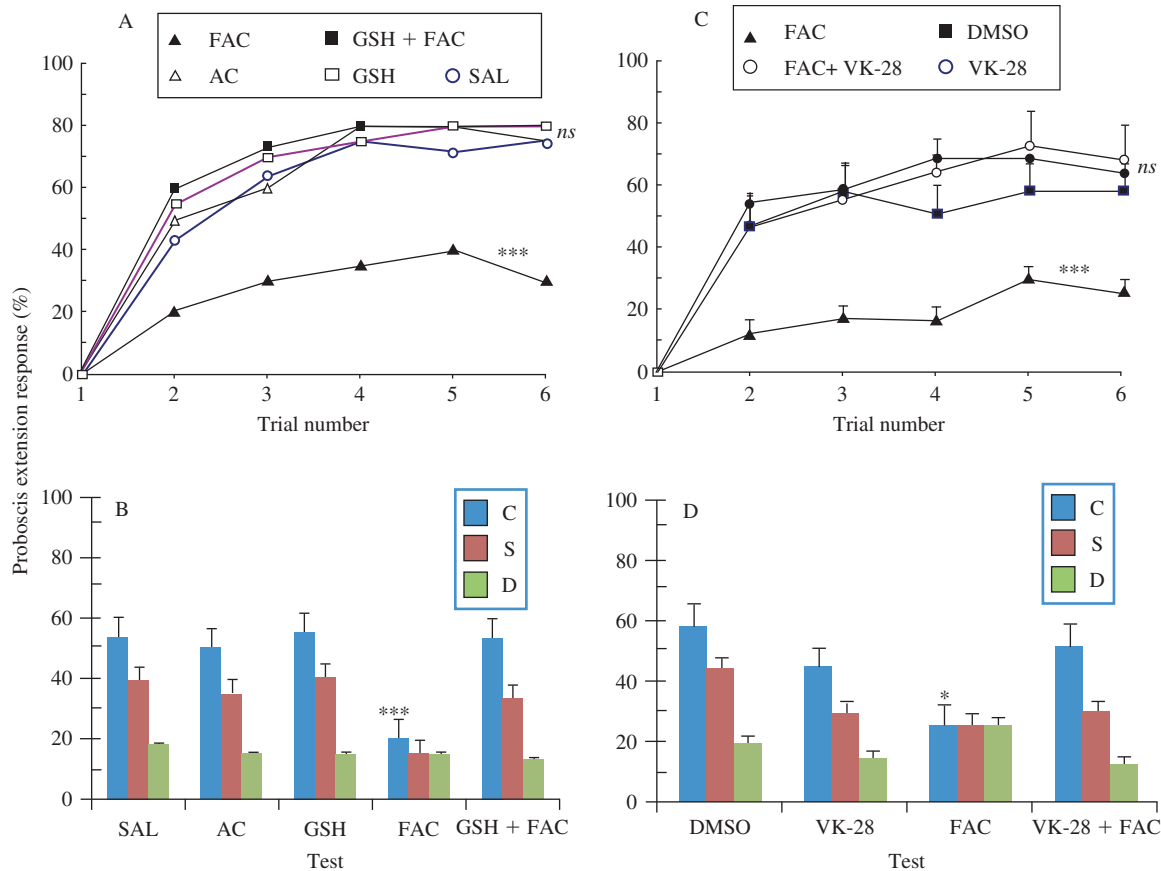


Fig. 21.4 Glutathione and VK-28 can reverse ferrous ammonium citrate (FAC)-mediated oxidative stress. (A) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either saline (SAL) or 2.5 mM reduced glutathione (GSH) in each antennal lobe. Two hours later, 4 nl of 500 μ mol/l ammonium citrate (AC) or 500 μ mol/l FAC were injected in each antennal lobe. Twenty-four hours later, subjects were conditioned with 1-octanol. (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with different odors (1-octanol, C; 1-hexanol, S; and geraniol, D) in randomized order. (C) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either dimethyl sulfoxide (DMSO) or VK-28 or DMSO + VK-28 in each antennal lobe 30 min before injection of FAC. Twenty-four hours later, subjects were conditioned with 1-octanol. (D) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, S, and D odors in randomized order. Asterisks indicate significant differences of respective points from control group (* $P = 0.05$, *** $P = 0.001$). *ns*, Not significant. This figure is modified from reference [40].

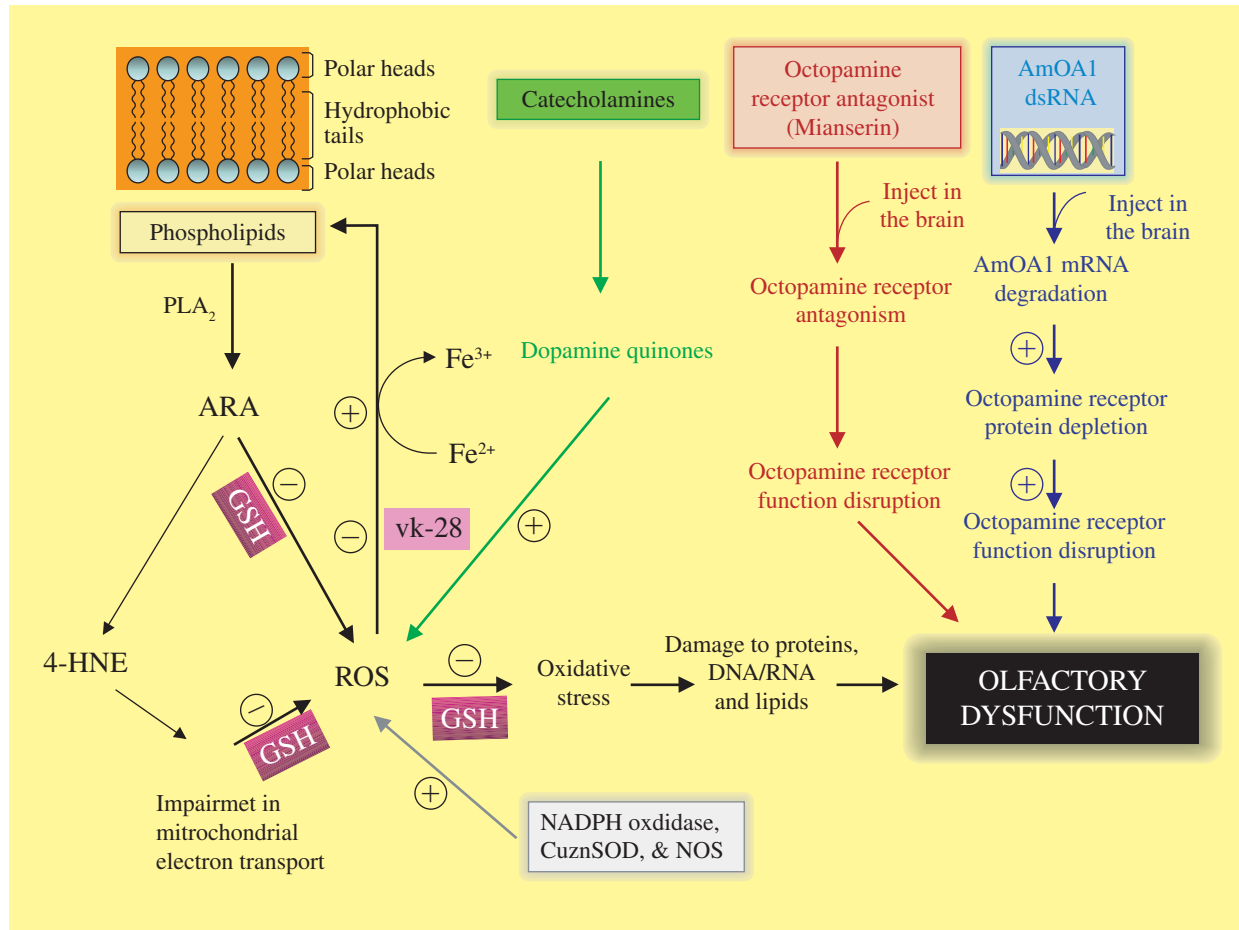


Fig. 21.5 Hypothetical molecular mechanism underlying reactive oxygen species (ROS)-mediated olfactory dysfunction in the honeybee brain. Iron-induced oxidative stress: Iron peroxidizes polyunsaturated fatty acids into peroxidized phospholipids, which are considered better substrates for phospholipase A_2 (PLA_2) than native phospholipids. PLA_2 catalyzes this reaction, forming arachidonic acid (ARA). The nonenzymatic oxidation of ARA results in the generation of 4-hydroxynonenal (4-HNE) that impairs mitochondrial electron transport, producing ROS. Increased ROS produces oxidative stress that damages proteins, DNA/RNA, and lipids, impairing olfactory processes (encoding, consolidation, and/or retrieval processes). Both octopamine receptor antagonism by mianserin (MAS) and octopamine receptor protein depletion by octopamine receptor double-stranded RNA (AmOA1-dsRNA) result in functional disruption of octopamine receptor, which leads to olfactory dysfunction. Oxidation of catecholamines forms quinones, which results in formation of ROS, leading to olfactory dysfunction. GSH protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins. VK-28 chelates excess iron from the system. Monoamine oxidase inhibitor (MAOI) inhibits monoamine oxidase enzyme and therefore prevents oxidation of catecholamines. ROS can also be produced by activation of NADPH oxidase.

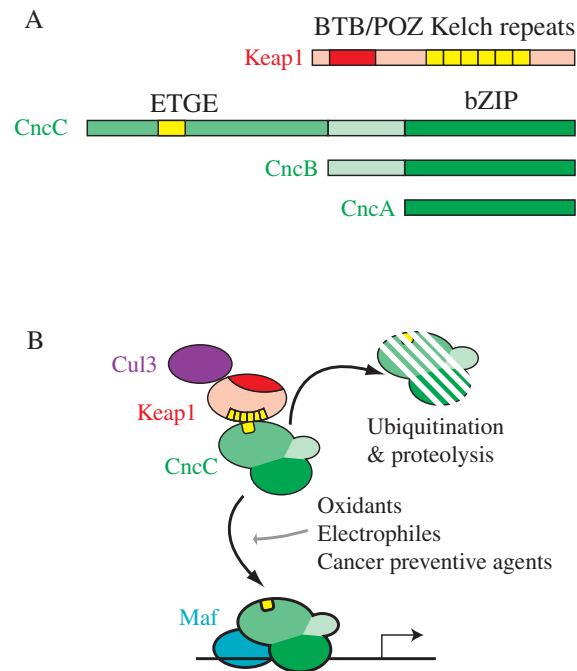


Fig. 22.1 Conservation and simplified illustration of the Keap1/Nrf2 pathway in *Drosophila*. (A) Nrf2 and Keap1 homologs are present in *Drosophila*. The fly *keap1* gene is predicted to encode a protein with high sequence similarity to its vertebrate Keap1 counterparts. Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. The *cnc* locus encodes three protein products, which all contain the bZIP region that mediates dimerization and DNA binding. The Nrf2 homolog is the longest isoform, CncC, which contains domains predicted to bind Keap1 such as the ETGE motif. (B) In basal conditions, Keap1 binds to CncC and inhibits its activity, likely through Cul3-mediated ubiquitination and proteasomal degradation. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents relieve this inhibition. Stabilized CncC then accumulates in the nucleus and transcriptionally activates a battery of cell-protective genes, likely in a dimer with the single small Maf protein of *Drosophila*. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

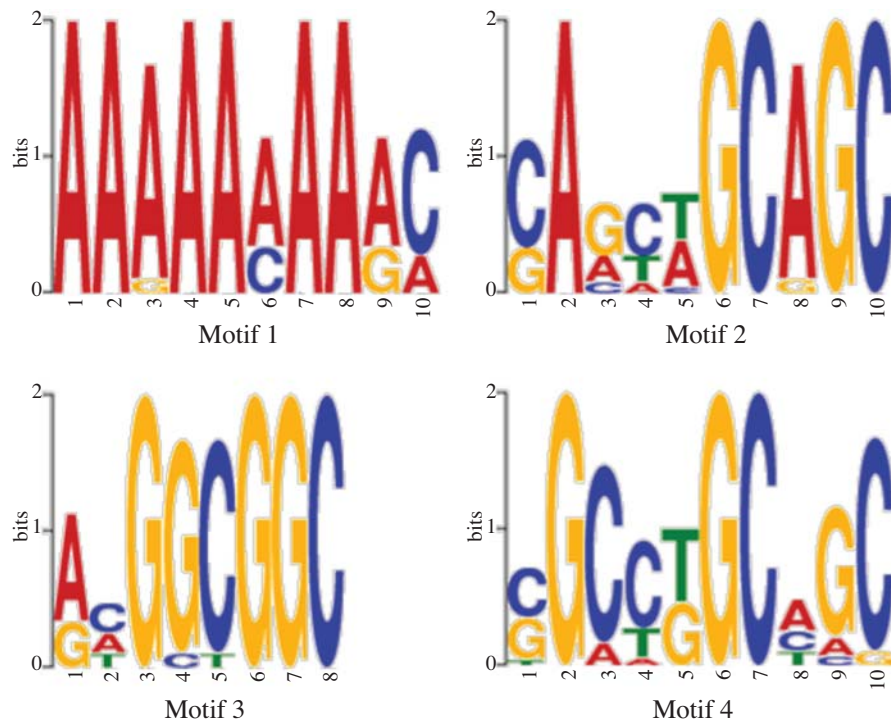


Fig. 23.4 Sequence LOGOS of the MEME motifs 1–4 displaying the probability of each base appearing at every possible position in the motif. The total height of the stack is the information content of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack.

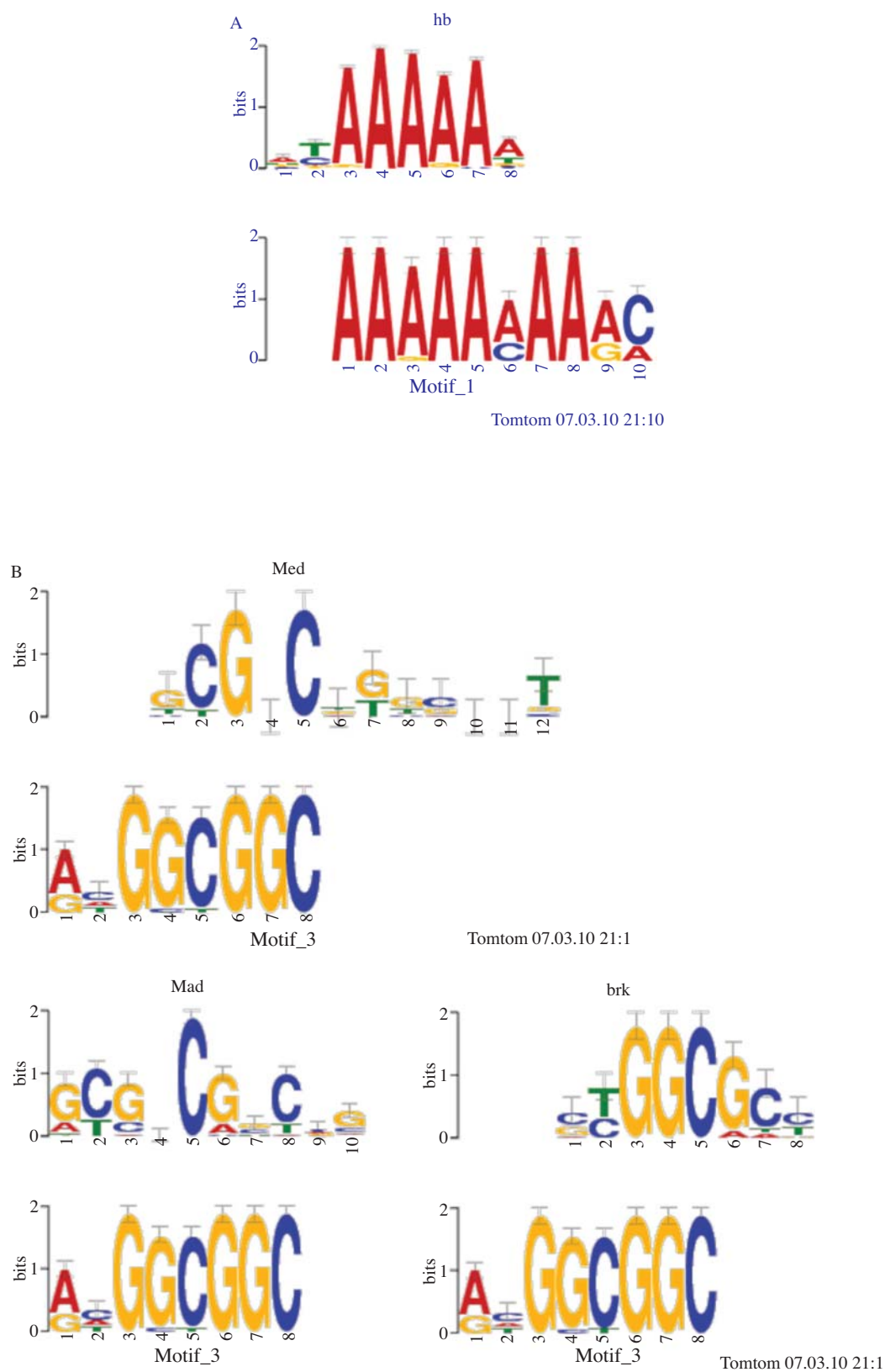


Fig. 23.5 TOMTOM output of the comparison of MEME motifs with existing motifs in the *Drosophila* database (FLYREG; Bergman and Pollard v2). Only statistically significant matches ($P < 0.001$) with a low false discovery rate ($q < 0.5$). (A) Motif 1. (B) Motif 3. (C) Motif 4.

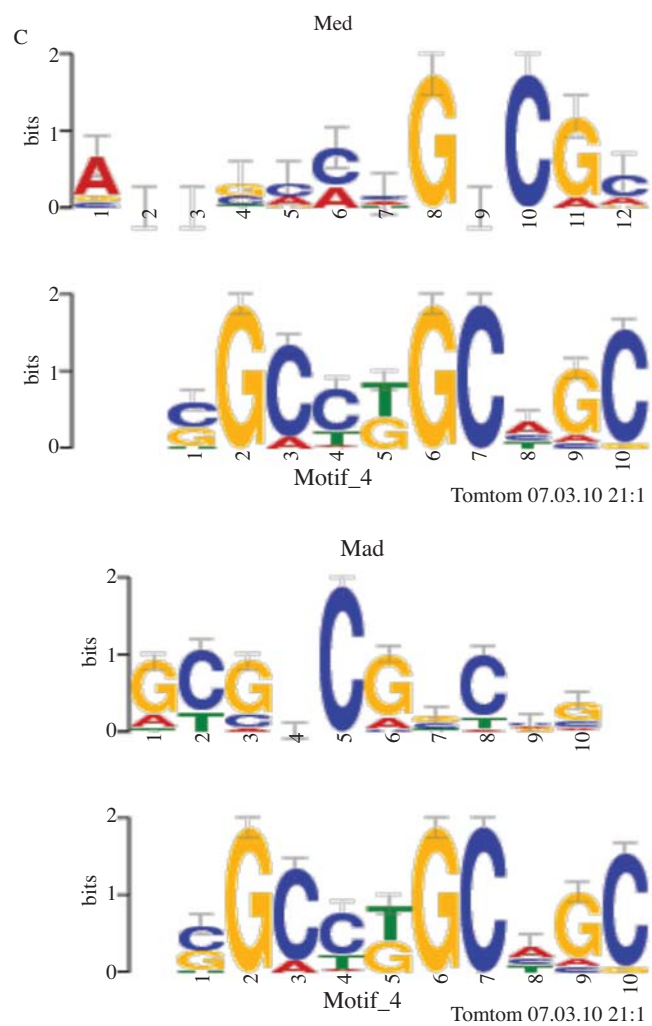


Fig. 23.5 (Continued)

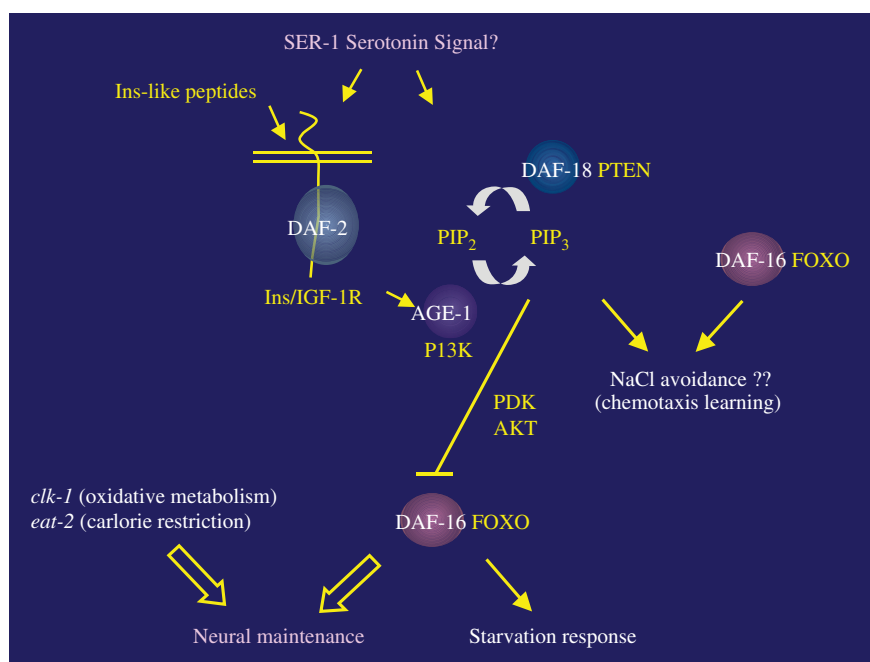


Fig. 25.3 A model for memory regulation by the insulin/IGF-1 pathway. It appears likely that the effects of life extension, or improved neuronal maintenance, lead to an increase in temperature-food association.

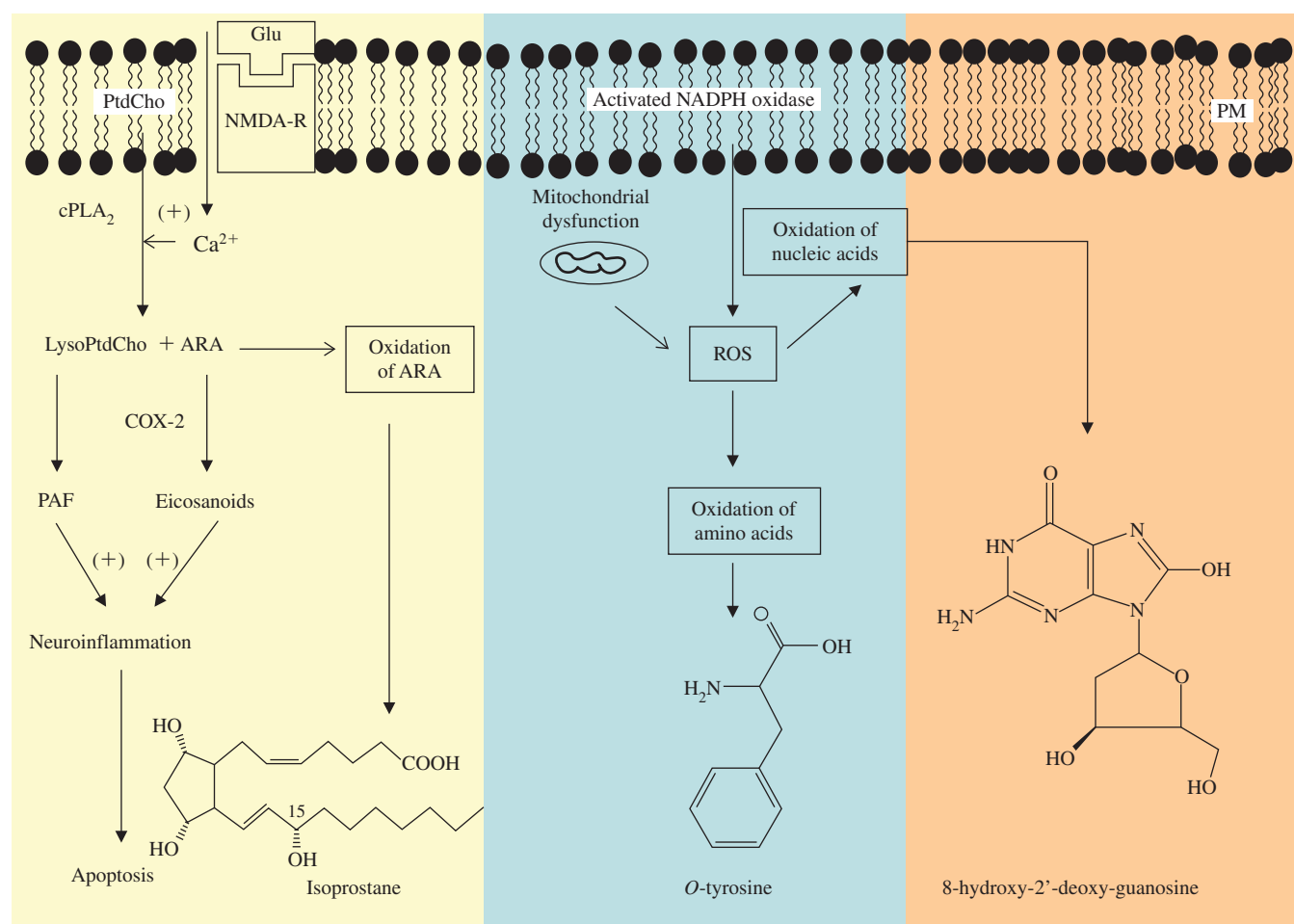


Fig. 27.2 Generation of biomarkers for oxidative stress in vertebrates. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; COX-2, cyclooxygenase; ARA, arachidonic acid; PAF, platelet-activating factor; ROS, reactive oxygen species.

PART I

OXIDATIVE STRESS IN VERTEBRATES

GENERATION OF REACTIVE OXYGEN SPECIES IN THE BRAIN: SIGNALING FOR NEURAL CELL SURVIVAL OR SUICIDE

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1.1 INTRODUCTION

Oxidative stress is a redox-sensitive process that occurs in the cell when antioxidant mechanisms are overwhelmed by the generation of reactive oxygen species (ROS), leading to oxidation of lipids, proteins, and DNA in ways that impair cellular function [1]. Thus oxidative stress is a threshold phenomenon characterized by a major increase in the amount of oxidized cellular components. ROS include superoxide anions, hydroxyl, alkoxyl, and peroxy radicals, and hydrogen peroxide, which are generated as by-products of oxidative metabolism, in which energy activation and electron reduction are involved. The chemical reactivity of ROS varies from the very toxic hydroxyl ($\cdot\text{OH}$) to the less reactive superoxide radical ($\text{O}_2^{\cdot-}$). The initial product, $\text{O}_2^{\cdot-}$, results from the addition of a single electron to molecular oxygen. $\text{O}_2^{\cdot-}$ is rapidly dismutated by superoxide dismutase (SOD), yielding H_2O_2 and O_2 , which can be reused to generate superoxide radical. In the presence of reduced transition metals, H_2O_2 , although less reactive than $\text{O}_2^{\cdot-}$, and highly diffusible, can be converted into the highly reactive hydroxyl radical $\text{HO}\cdot$. The tight regulation of ROS generation and removal makes fluctuations in their levels transient, a feature that is characteristic of second messengers. ROS may also act as an intracellular “rheostat,” closely modulating the activity of a discrete set of biochemical reactions, which contribute to cell proliferation, migration, and survival [2]. ROS not only

inactivate membrane proteins and DNA but also promote peroxidation of neural membrane polyunsaturated fatty acids associated with glycerophospholipids, enhance levels of ceramide, and facilitate the formation of hydroxyl/ketocholesterol levels (Fig. 1.1). These processes promote neurodegeneration through apoptosis [3–5]. The polyunsaturated fatty acids, which are located at the *sn*-2 position of glycerol moiety in the glycerophospholipid, are most susceptible to free radical attack at the α -methylene carbon in the alkyl chain of the fatty acid that is adjacent to the carbon-carbon double bond. Under aerobic conditions a polyunsaturated fatty acid with an unpaired electron undergoes a molecular rearrangement by reaction with O_2 to generate a peroxy radical. The peroxy radical captures hydrogen atoms from the adjacent fatty acids to form a lipid hydroperoxide. The lipid hydroperoxides thus formed are not completely stable *in vivo* and, in the presence of iron, can further break down to radicals that can propagate the chain reactions started by an initial free radical attack. The major sources of ROS are the mitochondrial respiratory chain, where $\text{O}_2^{\cdot-}$ is generated by electron leakage from complexes I and III of the electron transport chain (Fig. 1.2) [6, 7]. Microsomes and peroxisomes are also sources of ROS, primarily H_2O_2 , whereas immune cells such as neutrophils and macrophages possess oxygen-dependent mechanisms to fight against invading microorganisms. Enzymes, such as xanthine/xanthine oxidase, myeloperoxidase, cytochrome *P*450 in cell cytoplasm,

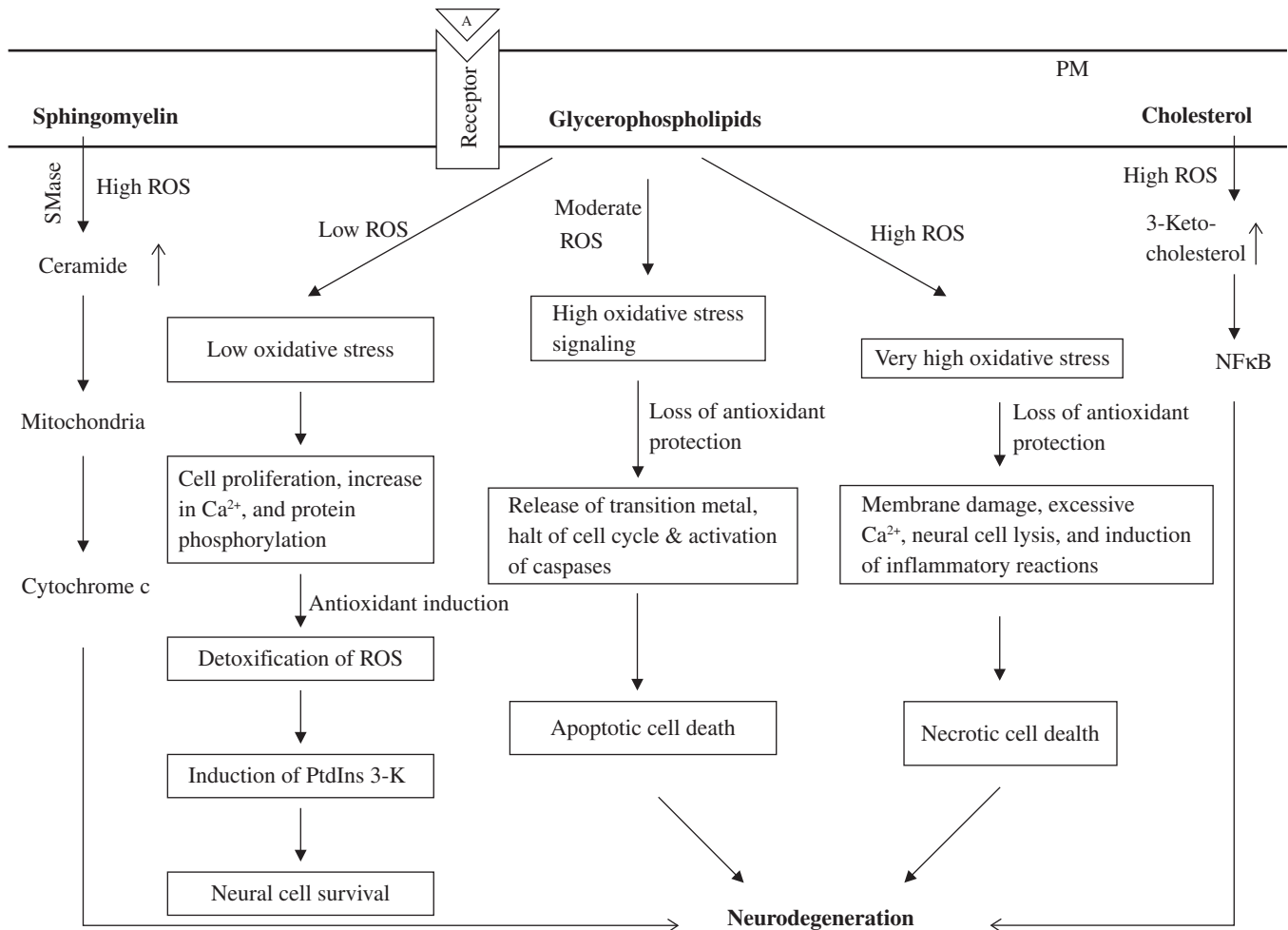


Fig. 1.1 Effect of reactive oxygen species (ROS) on lipid constituents (glycerophospholipid, sphingolipid, and cholesterol) of neural membranes. Low ROS levels promote neural cell survival, whereas high ROS levels promote neurodegeneration through apoptotic and necrotic cell death. PM, plasma membrane; NF- κ B, nuclear factor- κ B; SMase, sphingomyelinases.

COX, LOX, nitric oxide synthase, and NADPH oxidase contribute to ROS production in plasma membranes and mitochondria (Fig. 1.2). The presence of redox-active metals, such as iron and copper, also contributes to ROS generation. In the presence of Fe^{2+} and Fe^{3+} , HO^\bullet can be generated through the Fenton reaction or the Haber-Weiss reaction [7].

1.2 ROLE OF REACTIVE OXYGEN SPECIES IN NEURAL CELLS

As stated above, in brain ROS are generated during oxidative metabolism. ROS-mediated damage to neural membranes is accompanied by (a) changes in physico-chemical properties of neural membranes (microviscosity and fluidity) not only resulting in exchange of phospholipids between the two halves of the lipid bilayer but also

altering the orientation of optimal domains of receptors, enzymes, and ion channels; (b) changes in the number of receptors and their affinity for neurotransmitters; and (c) inhibition of ion pump operation and entry of K^+ and Ca^{2+} into neural cells resulting in changes in ion homeostasis. The presence of peroxidized glycerophospholipids in neural membranes may also induce a membrane-packing defect, making the *sn*-2 ester bond more accessible to the action of phospholipase A_2 (PLA_2) and the release of free arachidonic acid (ARA) or docosahexaenoic acid (DHA). ARA and DHA act as substrates for the synthesis of eicosanoids and docosanoids, respectively [3]. Lyso-phospholipid, the other product of PLA_2 -catalyzed reaction, not only induces detergent-like effects leading to further disorganization of neural membranes but also acts as substrate for platelet-activating factor (PAF) [8]. Emerging evidence suggests that enzymic and nonenzymic oxidation of polyunsaturated fatty acids

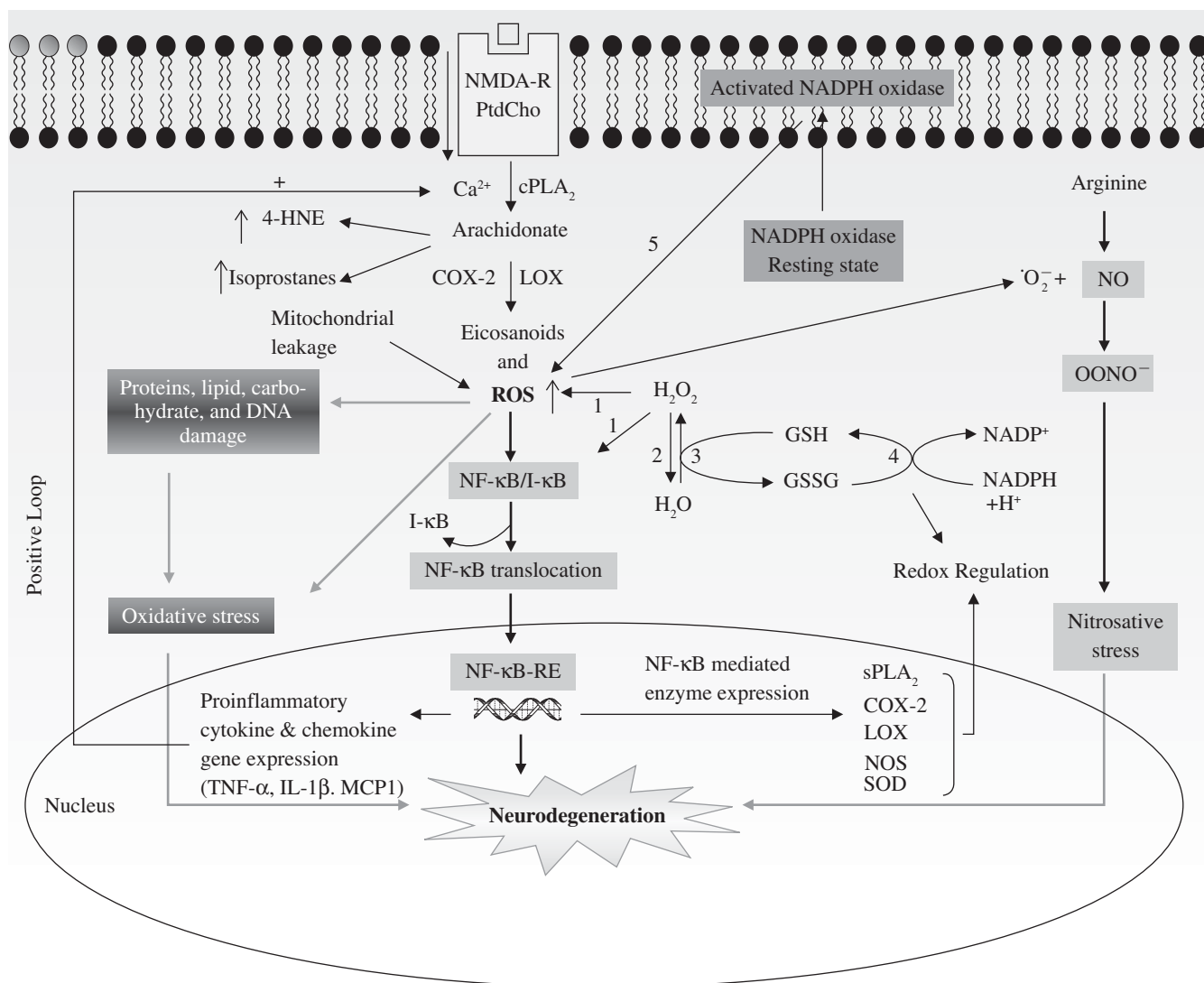


Fig. 1.2 Generation of reactive oxygen species (ROS) and enzymic and nonenzymic markers for oxidative stress. 1, Superoxide dismutase (SOD); 2, catalase; 3, glutathione peroxidase; 4, glutathione reductase; 5, NADPH oxidase. cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; H₂O₂, hydrogen peroxide; 4-HNE, 4-hydroxynonenal; NO, nitric oxide; OONO⁻, peroxynitrite. Activation of NF-κB by ROS leads to its translocation to the nucleus, where it facilitates the transcription of proinflammatory enzymes (sPLA₂, COX-2, NOS, and SOD) and proinflammatory cytokines (TNF-α and IL-1β). These cytokines upregulate activities of cPLA₂ and sPLA₂ through a positive loop mechanism in cytoplasm and neural membranes. Upward arrows indicate increase in levels of metabolites. (See color insert.)

leads to the formation and accumulation of ARA-derived eicosanoids, 4-hydroxy-2-nonenal (4-HNE), isoprostanes, isofurans, and isoketals and DHA-derived docosanoids, 4-hydroxyhexanal, neuroprostanes, neurofurans, and neuroketals that induce specific cellular dysfunction [3, 5, 9]. In addition, lipid peroxidation also leads to the generation of lipid hydroperoxides, which inhibit the recylation of phospholipids in neuronal membranes [10]. The detoxification of glycerophospholipid

hydroperoxides is accomplished through the combined enzymic activity of PLA₂ and reduction of the released fatty acid hydroperoxides with phospholipid hydroperoxide glutathione peroxidase [11–13]. The latter enzyme not only acts on membranes and reduces glycerophospholipid hydroperoxides to the nontoxic hydroxyl derivatives [14, 15] but reduces H₂O₂ to water to limit its harmful effects. Phospholipid hydroperoxide glutathione peroxidase is different from the classic glutathione

peroxidase, which mainly reduces H_2O_2 . The restoration of neural membrane integrity by the reaction catalyzed by phospholipid hydroperoxide glutathione peroxidase is achieved by the reinsertion of nonoxidized fatty acyl groups through the involvement of the deacylation/reacylation cycle [16]. Nonenzymically, ROS buildup can be prevented by vitamins E and C. These vitamins terminate lipid chain reactions involving peroxy radicals. In addition to being a cofactor of various antioxidant enzymes, GSH, which is the most abundant peptide in cells, performs many functions. The thioredoxin system is another important thiol antioxidant system consisting of thioredoxin (Trx) and thioredoxin reductase. Trx is a multifunctional selenoprotein containing two redox-active cysteines and a conserved active site (Cys-Gly-Pro-Cys) [17, 18]. Although many ROS are quenched by GSH, other thiol-containing proteins also participate in neutralizing ROS [19].

1.2.1 Modulation of Enzyme Activities, Transcription Factors, and Genes by ROS

ROS regulate activities of several enzymes in neural cells. Thus ROS not only modulate activities of protein tyrosine kinases, protein phosphatases, and mitogen-activated protein kinases [extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38 pathway] (Fig. 1.3) but also play an important role in regulating intracellular Ca^{2+} homeostasis and RhoA/Rho kinase signaling [20]. Low and moderate levels of ROS activate PtdIns 3-kinase signaling and promote cell survival. PtdIns 3-kinase/protein kinase B (Akt) transduces the signal for cell survival mainly through phosphorylation of target molecules by Akt. This results in the inactivation of proapoptotic proteins and activation of transcription factors that target the expression of antiapoptotic proteins. ROS increase vascular $[Ca^{2+}]_i$ by stimulating inositol trisphosphate-mediated Ca^{2+} mobilization, by increasing cytosolic Ca^{2+} accumulation through sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase inhibition, and by stimulating Ca^{2+} influx through Ca^{2+} channels. Increased ROS production enhances Ca^{2+} signaling and upregulates RhoA/Rho kinase, thereby altering vascular contractility and tone in the vasculature [21]. ROS also inactivate protein tyrosine phosphatases in a dose- and time-dependent manner [22, 23]. ROS activate metalloproteinases, and this stimulation is blocked by *N*-acetyl cysteine [24]. In addition, ROS modulate transcription factors (NF- κ B, HIF, CREB, AP-1, ATF2, A-1, CHOP-1, and E2F), modulate the cell cycle, and ion transport (Fig. 1.3). Although the molecular mechanisms underlying ROS-mediated alterations of kinases and transcription factors are not fully understood, it is becoming

increasingly evident that the regulation of stress-responsive proteins by ROS may be closely associated with the above alterations. ROS-mediated cellular changes involve (a) the direct effect of ROS on the kinase or transcription factor, which can alter conformation and activity, and (b) the effect of cysteine-rich, redox-sensitive proteins, which are associated with the regulation of stress-responsive proteins. Oxidative stress not only produces conformational changes in redox-responsive proteins but also facilitates the generation of dimers/multimers of these proteins. The redox-responsive proteins include thioredoxin and glutathione *S*-transferase [25]. Emerging evidence suggests that low levels of ROS induce minor changes in levels of Ca^{2+} , enzyme activities, transcription factors, cell cycle, and ion transporters, which support and maintain normal cell function through the tight regulation of diverse intracellular signaling networks. However, moderate and high levels of ROS can inflict damage to all subcellular organelles (e.g., mitochondria, endoplasmic reticulum, etc.), eventually leading to cell death.

The brain processes large amounts of O_2 in relatively small mass and has a high content of substrates available for oxidation in conjunction with low antioxidant activities making polyunsaturated fatty acids found in glycerophospholipids extremely susceptible to oxidative damage. In addition, neurons of certain regions of the brain, such as the hippocampus, may be particularly vulnerable to oxidative stress because of their low endogenous levels of vitamin E and glutathione relative to other brain regions. Such a depressed defense system may be adequate under normal circumstances. However, generation of high levels of ROS following acute neural trauma (stroke, spinal cord trauma, and traumatic brain injury) and neurodegenerative diseases, such as Alzheimer disease (AD), Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS), and low antioxidant defenses can predispose the brain to high oxidative stress leading to neuronal injury and death [3].

1.2.2 Modulation of Genes by ROS

In nonneural cells, ROS regulate many genes, including adhesion molecules and chemotactic factors, antioxidant enzymes, and vasoactive substances [2]. Some of these genes are associated with adaptive responses. This includes the induction of superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx) by H_2O_2 , supporting the view that newly synthesized protective proteins are needed for adaptive responses [26, 27]. Most redox-sensitive genes have been identified on the basis of their responsiveness to externally applied oxidant stress; only a few have been shown to be

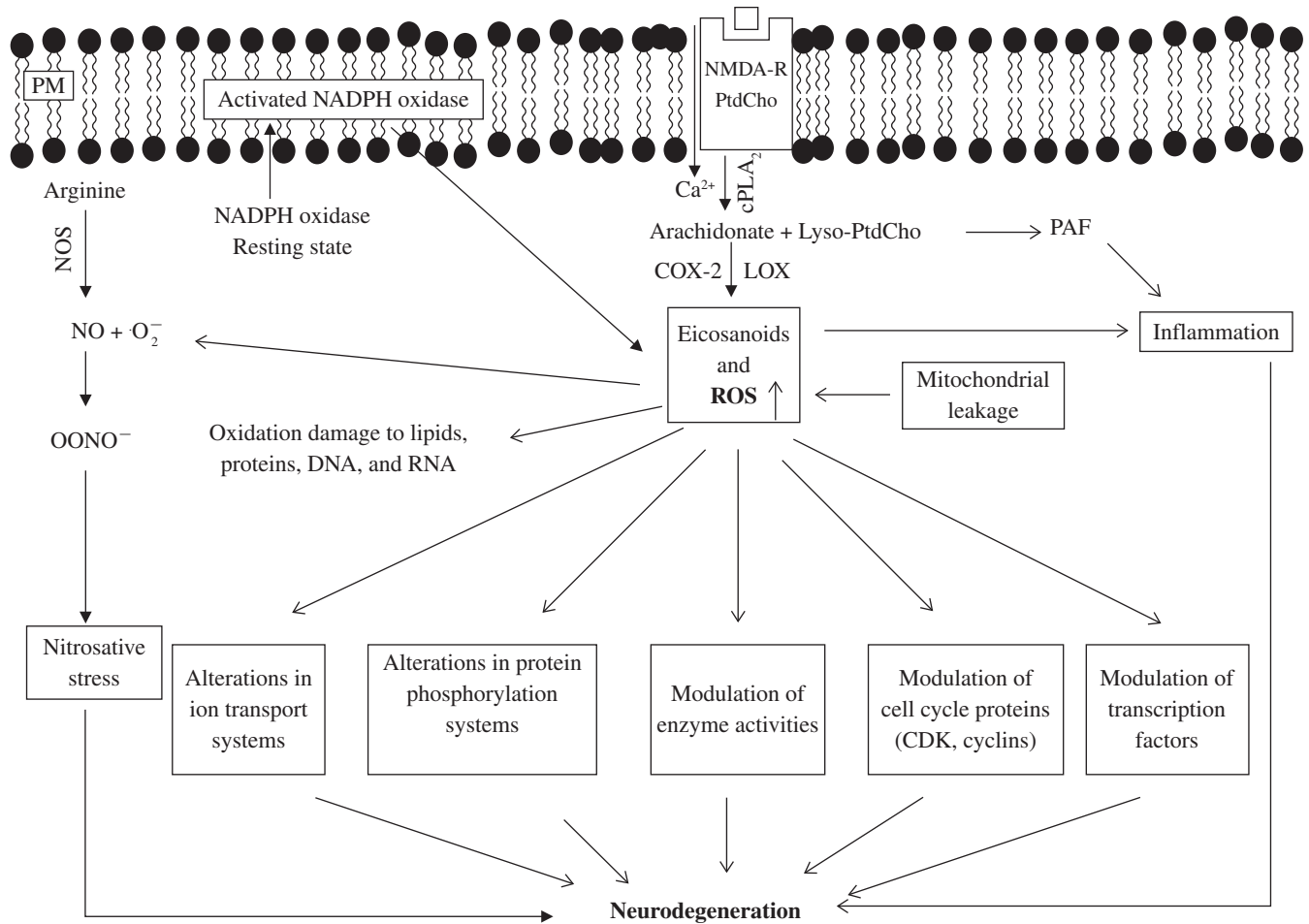


Fig. 1.3 Roles of reactive oxygen species (ROS) in the brain. cPLA₂, cytosolic phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; NO, nitric oxide; OONO⁻, peroxynitrite; PAF, platelet-activated factor. In brain, ROS are generated through mitochondrial dysfunction, ARA oxidation, and NADPH oxidase activation. Low ROS levels modulate ion transport, protein phosphorylation, enzymic activities, cell cycle, and transcription factors, but high ROS levels damage neural membrane lipids, protein, and nucleic acid.

downstream of an endogenous source of ROS, such as the NADPH oxidase. These include TNF- α - and lactosylceramide-mediated induction of intercellular adhesion molecule (ICAM-1) [28] and Ang II, PDGF, and TNF- α stimulation of monocyte chemotactic protein (MCP)-1 [29]. In contrast, stimulation of MCP-1 by IL-1 β in vascular smooth muscle cells (VSMCs) is not affected by antioxidants, suggesting that the control of gene expression by ROS is both stimulus- and tissue specific [30]. Neural and nonneural cells possess signaling pathways that can sense oxidative stress and launch adaptive responses that bolster the antioxidant defense networks. Accumulating evidence suggests that modulation of gene expression by ROS occurs at cellular and subcellular levels. Low ROS levels involve modulation

of some neuroprotective genes along with redox-sensitive transcription factors (AP-1 and Nrf2). These factors modulate genes for antioxidant response element (ARE), endogenous antioxidants, phase II detoxifying enzymes, and transporters. Nrf2 is a transcription factor that regulates the basal and inducible expression of a wide array of antioxidant genes. After phosphorylation and dissociation from the cytosolic protein Keap1, a scaffolding protein that binds Nrf2 and Cul3 ubiquitin ligase for proteasomal degradation, Nrf2 rapidly translocates to the nucleus, where it activates the ARE in the promoter region of many antioxidant genes [31]. Nrf2 activates transcription primarily through the formation of a dimer with a small musculoaponeurotic fibrosarcoma oncogene family of proteins (Maf) [32, 33]. The binding

of the small Maf-Nrf2 dimers to ARE sequences leads to a coordinated transcriptional activation of a battery of antioxidant enzymes and detoxifying proteins. This regulated adaptive response is called the “phase II detoxification response” [34]. Activation of Nrf2 not only increases the abundance of thioredoxins and glutathione-synthesizing enzymes and glutathione *S*-transferases but also enhances the expression of molecular chaperones, proteasome subunits, and various other cytoprotective proteins [35]. Expression of the Nrf2-dependent proteins is critical to maintaining cellular redox homeostasis through elimination of toxins [2]. Modulation of other genes involves translocation of a specific transcription factor NF- κ B, which facilitates expression of proinflammatory enzymes, chemokines, and cytokines. The mechanism by which cytokines (TNF- α) induce neurodegeneration appears to be related not only to the depletion of GSH but also to the redox-dependent generation of ceramide from sphingomyelin, formation of 4-HNE and isoprostane from membrane glycerophospholipids, and generation of hydroxyl- and ketocholesterol from cholesterol [5, 6]. Hydroperoxy fatty acids and H₂O₂ promote the expression of c-Fos and Jun 2 proteins that form heterodimers and activate AP-1 [36]. Activation of nitric oxide synthase (NOS) during oxidative stress generates NO[•], an important signaling molecular and vasodilator. NO[•] increases the transcription of I κ -B, the inhibitory factor that binds NF- κ B and facilitates its retention in the cytoplasm [37]. The turnover of I κ -B protein is also oxidant sensitive, and antioxidants can block agonist-mediated stimulation of I κ -B phosphorylation and degradation [2, 37]. Conversely, H₂O₂ increases translocation of NF- κ B to the nucleus, where it facilitates the transcription of responsive genes [38]. In addition, several other mammalian transcription factors are directly modified by ROS or by reducing proteins that modify cysteine residues involved in DNA binding [2]. These transcription factors include AP-1, NF- κ B, and hypoxia-inducible factor-1 (HIF-1) [2, 39, 40]. Both c-Fos and c-Jun contain a conserved cysteine in a basic motif that, when oxidized, interferes with the interaction of these proteins with AP-1 consensus sequences. Conversely, if c-Fos/c-Jun heterodimers are complexed with AP-1, they cannot be oxidized [39]. The oxidation state of these important proteins is modulated by redox factor-1 [1], a protein that, in cooperation with thioredoxin, facilitates the cycling of the critical cysteines between reduced and oxidized forms [2, 39]. Thioredoxin also modulates HIF-1-dependent transcription [40] and modifies the DNA binding and transcriptional activity of NF- κ B by reducing cysteine 62 [41]. Collectively, these studies indicate the importance of the nuclear redox state in regulating ROS-mediated gene expression [2].

1.2.3 Modulation of Long-Term Potentiation, Cognition, and Memory Formation by ROS

It is well known that hippocampus is involved in synaptic plasticity associated with cognitive function and learning and memory. This region is highly susceptible to oxidative stress [42]. Long-term potentiation (LTP) is defined as a long-lasting increase in synaptic efficacy following high-frequency stimulation of afferent fibers. Treatment of hippocampal slices with H₂O₂ at millimolar concentrations produces oxidative stress [43] and inhibition of LTP, whereas micromolar concentrations of H₂O₂ enhance LTP [44, 45]. The action of H₂O₂ is mediated through the release of calcium ions from internal stores, modulating the activity of specific calcium-dependent protein phosphatases, PLA₂, and phospholipase C (PLC). These enzymes modulate synaptic plasticity. The above observations are supported by studies in aged mice overexpressing extracellular SOD. These mice perform better in a water maze memory task than aged control mice [46]. It is also reported that an increase in SOD activity, which impairs LTP, is caused by a secondary increase in H₂O₂ levels and catalase reverses the effects of SOD. The molecular mechanism associated with H₂O₂-mediated impairment of LTP is not fully understood. However, it is becoming increasingly evident that serine/threonine phosphatases (PP2A) contribute to the impairment of LTP [45, 47]. The ketogenic diet (high-fat and low-carbohydrate with anticonvulsant), which induces ketonemia, not only downregulates PP2A activity and expression of this enzyme but also prevents oxidative stress-mediated impairment of LTP by inhibiting PP2A [48–50]. It is proposed that oxidative stress-mediated impairment of hippocampal LTP is associated with low levels of ROS production, changes in synaptic plasticity, and activation of PP2A, and that ketone bodies prevent this impairment of LTP through the inhibition of PP2A. The regulation of synaptic activity by ROS is not confined to hippocampal synapses. A series of studies have indicated that H₂O₂ modulates dopamine release in dorsal striatum through a ROS sensor on potassium channels that control the excitability of the dopamine-releasing neurons [51]. Emerging evidence suggests that the signal transduction network associated with synaptic plasticity involves many players, including protein kinases, phosphatases, phospholipases, transcription factors, and other Ca²⁺-dependent enzymes [52], which contribute to the generation of ROS.

1.2.4 Modulation of Cell Death by ROS

As mentioned above, the brain consumes large quantities of oxygen relative to its contribution to total body

mass. This, together with low levels of vitamin E, glutathione, and lipoic acid and low activities of SOD, catalase, and peroxidase, places the brain at the risk for damage mediated by ROS [6]. ROS generation through mitochondrial dysfunction gradually disrupts the intracellular calcium homeostasis, which modulates neuronal excitability and synaptic transmission, making neurons more vulnerable to additional oxidative stress, and leads to neurodegeneration. Among neural membrane components lipids are most susceptible to oxidative modification. Lipid peroxidation produces lipid radicals, which can further attack the subsequent lipid molecules and propagate through a chain reaction. Lipid peroxidation leads to the formation of a number of aldehyde by-products, including malondialdehyde (MDA), 4-HNE, and acrolein. The most abundant aldehydes are 4-HNE and MDA, while acrolein is the most reactive. In addition, stimulation of PLA₂s, sphingomyelinases, and cytochrome P450 hydroxylases produces high levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators, which support inflammatory processes leading to neural cell death in the brain [5, 6]. The highly reactive OH[•], generated through the Fenton reaction, and ONOO⁻ formed from the reaction between O₂^{•-} and nitric oxide (NO[•]), target protein components of neural membranes. Irreversible protein oxidation includes nitrosylation of cysteine sulfhydryl groups, tyrosine, methionine, and tryptophan by ONOO⁻. Nitration of tyrosine residues may inhibit its phosphorylation or adenylation, important for protein function [53]. Severe oxidative stress can induce disulfide bond-mediated protein cross-linkage or secondary oxidative modifications such as adduct formation between oxidized proteins and lipid peroxides or glycation products, leading to accumulation of damaged proteins and cell death [54]. Some protein modifications, such as phosphorylation, are reversible modifications that can be overcome by specific enzymes (protein phosphatases) that cause a protein to “revert” back to its original protein structure, while other protein modifications, such as protein nitration and HNE-mediated modification (4-HNE-histidine and glutathione-4-HNE Michael adducts), are irreversible. Oxidative modification of proteins may induce alterations in the structure of proteins with subsequent loss of normal physiological cell functions leading to cell death.

Compared with lipids and proteins, neural cell DNA is less susceptible to oxidative modifications because of its double-helix structure and the protective shield from histone and other coating proteins. However, under severe oxidative stress nuclear DNA damage is also oxidized with the generation of 8-hydroxy-2-deoxyguanosine (8-OHdG) [55]. Collective evidence suggests that ROS-mediated alterations in neural membrane

components, generation of high levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators, modifications of proteins and DNA, loss of Ca²⁺ homeostasis, and induction of mitochondrial dysfunction may result in neural cell death.

1.3 ROS-MEDIATED SURVIVAL SIGNALING IN NEURAL CELLS

The cellular response to ROS depends not only on their concentration but also on their chemical nature. Low concentrations of ROS do not cause cell death but instead induce an adaptive and survival response to the oxidative stress through modulation of proliferation, synaptic plasticity, gene transcription, and neuronal excitability (Fig. 1.4). Adaptive and survival responses are modulated by cellular Ca²⁺ gradient. In neural cells, maintenance of Ca²⁺ gradients requires reduction in ATP level, which is associated with generation of ROS through respiratory control mechanisms. The selective oxidation of calmodulin (a Ca²⁺ binding protein) and alteration in Ca²⁺-ATPase (a Ca²⁺-dependent enzyme associated with efflux of Ca²⁺) activity during oxidative stress may represent an adaptive response to oxidative stress that functions to downregulate energy metabolism and the associated generation of ROS. During oxidative stress, enhanced sensitivity of Ca²⁺ binding proteins is closely associated not only with modulation of signal transduction processes but also with intracellular energy metabolism, supporting the view that the selective oxidation of critical signal transduction proteins may represent a regulatory mechanism that functions to minimize the generation of ROS through respiratory control. Thus decrease in the rate of ROS formation, in turn, may promote cellular survival under conditions of low oxidative stress, when ROS overwhelm cellular antioxidant defense systems, by minimizing the nonselective oxidation of a range of lipid, proteins, and nucleic acids [56, 57]. In addition, ROS may function as signaling molecules that fine-tune neural cell metabolism through the selective oxidation of Ca²⁺ binding proteins in order to minimize widespread oxidative damage and protein aggregation. Formation of low ROS levels also minimizes protein oxidation, which promotes intracellular repair mechanisms that function to eliminate damaged and partially unfolded proteins. Since the rates of protein repair or degradation compete with the rate of protein aggregation, the modulation of intracellular Ca²⁺ concentrations and energy metabolism through the selective oxidation of critical signal transduction proteins (Ca²⁺ binding proteins) maintains cellular function by minimizing protein aggregation [58]. Furthermore, ROS, specifically H₂O₂, are also essential for

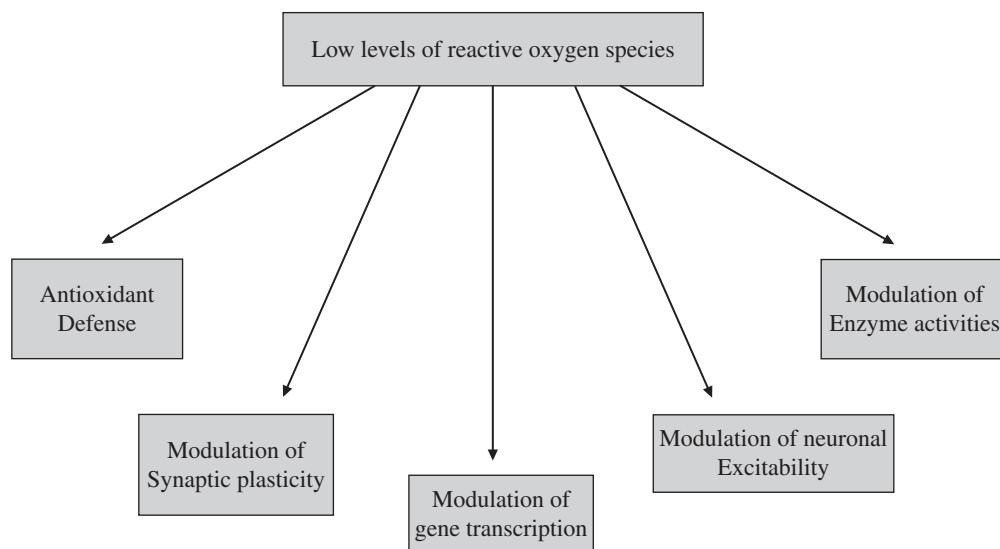


Fig. 1.4 Modulation of neural cell survival by low levels of ROS generated during normal metabolic conditions.

growth factor-mediated signal transduction, mitochondrial function, and maintenance of normal thiol redox-balance. These processes are closely associated with neural cell survival.

ROS activate both acidic and basic sphingomyelinases (SMases). The activation of SMases results in generation of ceramide. Low levels of this lipid mediator stimulate and modulate signaling pathways involved in the regulation of cell viability, differentiation, growth, and survival [59]. However, excessively high levels of ceramide can trigger apoptosis through the release of cytochrome *c*. Ceramides generated in response to membrane-associated oxidative stress have been implicated in the dysfunction and death of cells in neurotraumatic and neurodegenerative diseases [5].

1.4 ROS-MEDIATED INJURY IN NEURAL CELLS

High ROS levels induce neurodegeneration through apoptotic and necrotic cell death. The reaction between high ROS levels and proteins leads to a chemical cross-linking of membrane proteins and phospholipids resulting in alterations in membrane-bound enzymes and reduction in membrane unsaturation [60]. Alterations in activities of membrane-bound enzymes and depletion of unsaturation in membrane lipids are associated not only with decrease in activities of membrane-bound enzymes but also with decreased membrane fluidity and altered activities of ion channels and receptors [6, 61]. Oxidative damage to cellular proteins along with the loss of calcium homeostasis contributes to protein aggregation and deposition, a process that occurs

in neurodegenerative diseases [58]. In addition, high levels of 4-HNE generated during severe oxidative stress contribute to neurodegeneration by forming adducts with sulfhydryl groups (thiols) on proteins involved in neurotransmission [4, 62]. Moreover, in the presence of metal ions, such as Fe^{2+} and Cu^{2+} , H_2O_2 can be further transformed into hydroxyl radical ($\bullet\text{OH}$) through the Fenton reaction. Hydroxyl radicals can attack polyunsaturated fatty acids in membrane phospholipids, forming the peroxy radical ($\bullet\text{ROO}$), and then propagate the chain reaction of lipid peroxidation. Furthermore, high levels of ROS modulate the expression of genes responsible for modulating activities of cytokines and chemokines [3, 6]. Neurons are most susceptible to ROS-mediated oxidative injury. ROS also contribute to brain damage by activating a number of cellular pathways resulting in the expression of stress-sensitive genes and proteins associated with oxidative injury [63]. ROS-mediated injury to astrocytes induces apoptosis-like cell death through a caspase-3-independent mechanism [63]. In reactive microglia, activation of NADPH oxidase orchestrates the generation of superoxide, which is converted into O_2 and H_2O_2 by SOD.

Under severe oxidative stress, high levels of NO^\bullet are formed through enzymic oxidation of L-arginine to citrulline. $\text{O}_2^{\bullet-}$ reacts with NO^\bullet to form peroxynitrite (ONOO^-), a strong oxidant that can initiate lipid peroxidation and formation of nitrotyrosine in proteins (Fig. 1.5). This metabolite not only inhibits enzymes of the mitochondrial respiratory chain and inactivates glyceraldehyde-3-phosphate dehydrogenase, but also inhibits membrane Na^+/K^+ -ATPase and inactivates sodium channels in the membrane [64]. In addition, S-nitrosylation or covalent reaction of NO with specific

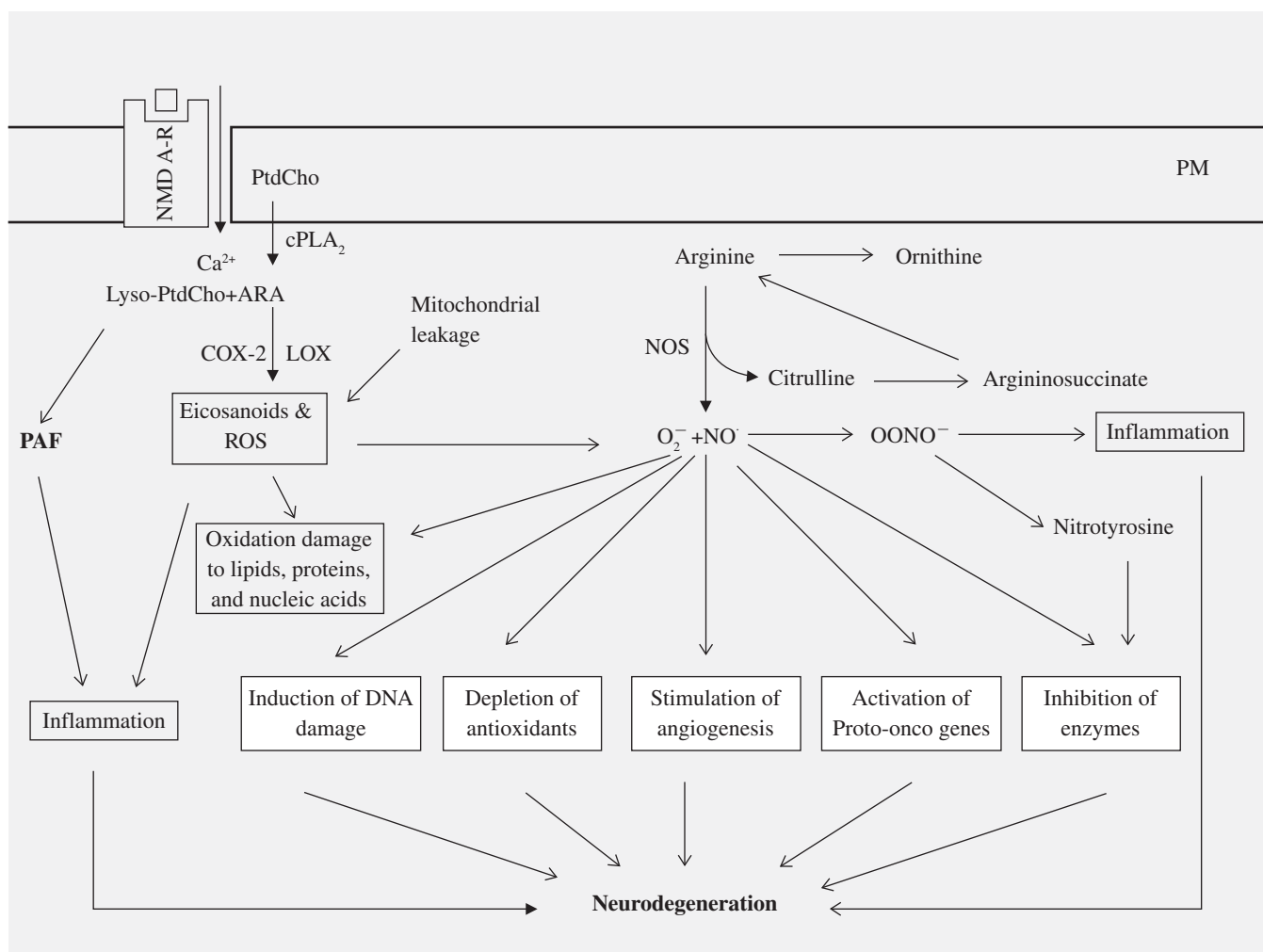


Fig. 1.5 Generation of nitric oxide (NO^\bullet) and its roles in signal transduction processes. cPLA₂, Cytosolic phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; OONO⁻, peroxynitrite and PAF, platelet-activating factor.

protein thiol groups represents one mechanism contributing to NO^\bullet -mediated protein misfolding and neurotoxicity [65, 66]. Although nitrosative stress has long been considered as a major mediator of neurodegeneration, the molecular mechanism of how NO^\bullet can contribute to neurodegeneration is not fully established. It has been suggested recently that nitration and nitrosylation of proteins contribute to the neurodegenerative process by inducing protein aggregation [66–68]. Under severe oxidative and nitrosative stress, the activation of NAD^+ -consuming enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is another likely mechanism for NO^\bullet -mediated energy failure and neurotoxicity. Although under mild oxidative stress the activation of PARP-1 is a repair process for neuronal protection, under high oxidative stress it causes neuronal energy compromise leading to neurodegeneration [6, 69]. As stated above, oxidative

stress activates both acidic and basic SMases and promotes the generation of ceramide [59]. This lipid mediator at high levels triggers apoptosis. Ceramides generated in response to membrane-associated oxidative stress are implicated in cell death in neurotraumatic and neurodegenerative diseases [5]. Although the molecular mechanism of ceramide-mediated apoptosis is not fully understood, ceramide has been reported to modulate the opening of the mitochondrial permeability transition pores (PTPs), which disrupts the transmembrane potential, thus causing the release of cytochrome *c* and the generation of hydrogen peroxide. These molecules induce the release of APAF-1 and caspase-3 activation, leading to apoptotic cell death. In addition, ceramide also induces changes in the expression of the Bcl-2 family of proteins by activating specific transcription factors such as NF- κ B and c-Jun [70]. Similarly, neural

membrane cholesterol under oxidative stress is transformed into hydroxy- and ketocholesterols through the action of cytochrome *P*450 hydroxylases. Generation of hydroxy- and ketocholesterols in neural cells also facilitates neurodegeneration through the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), the release of cytochrome *c*, and activation of caspase-3 [5].

At high levels, ROS-mediated hydroxylation of nucleic acid bases generates 8-OHdG from deoxyguanosine and alters DNA. DNA alterations are associated with aging, glutamate toxicity, and Alzheimer disease (AD) [3]. Upon DNA repair, 8-OHdG is excreted in the urine. Urinary 8-OHdG not only is a biomarker of generalized cellular oxidative stress but also is a risk factor for neurodegenerative diseases, various types of brain and visceral cancers, atherosclerosis, cardiovascular diseases, hypertension, and ischemia-reperfusion injury. Under low oxidative stress moderate DNA damage triggers cell-cycle arrest and initiates DNA-repair processes that ensure DNA integrity. However, if the intensity of ROS-mediated oxidative stress is high, then DNA repair does not occur and the neural cell dies by either apoptosis or necrosis [71]. 4-HNE forms Michael adducts with deoxyguanosine, yielding four diastereomeric 1,N(2)-dG adducts (6*R*, 8*S*, 11*R*), (6*S*, 8*R*, 11*S*), (6*R*, 8*S*, 11*S*), and (6*S*, 8*R*, 11*R*) with 8-hydroxyl and 6-(1-hydroxyhexyl) in the *trans* configuration [72]. These adducts may interfere with DNA replication and transcription, thereby contributing to the etiology of diseases associated with oxidative stress [73].

Generation of ONOO[−] also triggers DNA damage-including DNA strand breakage and base modification. ONOO[−] activates the nuclear enzyme PARP, resulting in energy depletion and apoptosis/necrosis of cells. ONOO[−]-modified DNA may also lead to the generation of autoantibodies in various autoimmune disorders such as systemic lupus erythematosus (SLE). In chronic inflammatory diseases, ONOO[−] formed by phagocytic cells may cause damage to DNA, generating neoepitopes leading to the production of autoantibodies [74]. Emerging evidence suggests that severe oxidative stress can cause mutations and epigenetic perturbation by damaging DNA and proteins that modify chromatin.

1.5 CONCLUSION

Oxidative stress refers to cytotoxic consequences caused by oxygen free radicals generated in a cell by processes that utilize molecular oxygen. The major sources of ROS include the mitochondrial respiratory chain, xanthine/xanthine oxidase, myeloperoxidase, cytochrome *P*450, COX, LOX, and NADPH oxidase. The presence of redox-active metals, such as Fe²⁺ and Cu²⁺, also

contributes to ROS generation. ROS-mediated activation of transcription factors (AP-1, NF- κ B, HIF-1) results in their translocation to the nucleus, leading to the transcription of genes involved in cell growth regulatory pathways. Although a decade ago the traditional view was that oxidative stress causes cellular damage, studies from the past several years indicate that low levels of ROS are needed for signal transduction processes associated with synaptic plasticity, memory formation, and gene expression associated with cell survival. The emerging view is that ROS can either enhance neural cell survival or promote cell death, depending on the magnitude and duration of the oxidative stress, genetic background, and redox states of the cells [75]. Generation of ROS not only serves as a stimulus for triggering stress-response induced signal-transduction pathways but also can modulate neural cell death/survival through direct oxidative modifications of neural membrane components and generation of lipid mediators. Under normal conditions, the balance between generation and elimination of ROS ensures the proper maintenance of neural cell metabolism and other functions. The final decision of whether the neural cell survives or dies is the result of the overall outcome of the integration of signals from redox-sensitive factors, levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators, and other regulatory mechanisms [75]. Accumulation of oxidative damage products (lipid mediators) and failure of cells to neutralize ROS-mediated stress may result in excessive cell death as occurs not only in neurotraumatic and neurodegenerative diseases but also in normal aging.

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FREE RADICALS, SIGNAL TRANSDUCTION, AND HUMAN DISEASE

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2.1 INTRODUCTION

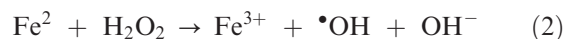
Free radicals are reactive species containing one or more unpaired electrons most often derived from oxygen (reactive oxygen species, ROS) or from nitrogen (reactive nitrogen species, RNS) [1]. ROS and RNS in living systems are generated by various enzymes, for example, by NAD(P)H oxidase or NO synthase (NOS). Beneficial effects of free radicals occur under low to moderate physiological concentration of radicals and maintain important physiological processes such as defense against infection and activation of various signaling pathways. Overproduction of free radicals as a consequence of, for example, the mitochondrial transport chain or overstimulated NAD(P)H results in oxidation stress that can be a mediator of damage to cell structures including lipids, proteins, and DNA [2].

Molecular oxygen has unique electronic properties; it contains two unpaired electrons on antibonding π^* orbitals possessing parallel spins and is itself a radical species. The addition of one electron originating, for example, from the mitochondrial electron transport system to the oxygen molecule leads to formation of superoxide anion radical $O_2^{\bullet-}$ ($O_2 + e^- \rightarrow O_2^{\bullet-}$) [3]. Superoxide radical is considered the “primary” upstream radical of the radical chain reactions in living systems, which can further react with other substrates to form “secondary” radicals. Superoxide radical is removed from the site of its action by a dismutation reaction [4].



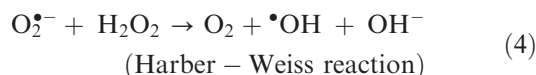
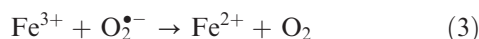
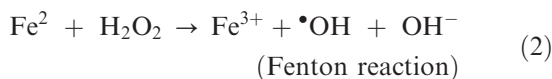
The dismutation reaction is catalyzed by the SOD enzyme working in conjunction with hydrogen peroxide-depleting enzymes such as catalases and glutathione peroxidases.

Under physiological conditions, redox-active metals are sequestered and cells contain only very limited amounts of free metal ions. Disruption of metal ion homeostasis leads to a state in which the concentration of free or unbound metals is elevated. Free metals can take part in catalytic decomposition reactions such as iron-catalyzed decomposition of hydrogen peroxide according to reaction [5]



The hydroxyl radical ($\bullet OH$) formed by this reaction has a half-life in aqueous environment of less than 1 ns and is one of the most reactive radicals occurring in biological systems. When produced in vivo it has a great ability to react at the site of its formation with neighboring biomolecules.

The reaction of superoxide radical with hydrogen peroxide can be described as the Haber–Weiss reaction, which is an overall reaction of the Fenton reaction (2) and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen [6]



Probably the most important RNS acting in biological systems is nitric oxide (NO^\bullet) generated by NOSs [7]. NO^\bullet is an important signaling molecule participating in various important physiological processes involving regulation of blood pressure, smooth muscle relaxation, regulation of immune system, neurotransmission, and other processes [8]. NO^\bullet contains one unpaired electron on an antibonding $2\pi_y^*$ orbital and therefore is a radical. NO^\bullet and $\text{O}_2^{\bullet-}$ can react together to form an oxidant molecule, peroxynitrite [9]



Under in vivo conditions peroxynitrite can react with carbon dioxide, forming an nitrosoperoxy carbonate (ONOOOCO_2^-). Peroxynitrite is oxidizing molecule causing oxidation of lipids and DNA fragmentation.

Under the conditions of imbalance between production and elimination of ROS and RNS termed oxidative stress, the organism uses various lines of defense. The first line of defense against deleterious action of free radicals is represented by antioxidant enzymes involving superoxide dismutase and catalase [10]. The second line of defense is represented by the small-molecular-weight antioxidants-vitamins, including vitamin C, vitamin E, carotenoids, lipoic acid, and others. Their structural properties allow them to donate an electron to a free radical and neutralize it.

2.2 REDUCTION, OXIDATION, AND THE THERMODYNAMICS OF FREE RADICAL REACTIONS

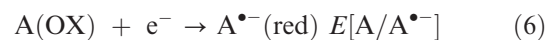
Oxidation and reduction reactions are called redox reactions and represent the basis for numerous biochemical mechanisms. When discussing redox reactions in biological systems, instead of the terms “reductant” and “oxidant,” it is more appropriate to use the terms “antioxidant” and “prooxidant,” respectively.

Free radical reactions are governed by the thermodynamic principles [11]. Thermodynamic properties of free radicals vary significantly, ranging from those capable of strong oxidation (e.g., reactive and damaging hydroxyl radical) to those capable of strong reduction (antioxidants such as vitamin C, glutathione, and others). It is

TABLE 2.1 Half-Cell reduction potentials of selected couples (pH = 7)

Couple	E^0/mV
$\bullet\text{OH}, \text{H}^+/\text{H}_2\text{O}$	+2310
$\bullet\text{OOH}, \text{H}^+/\text{H}_2\text{O}_2$	+1060
$\bullet\text{OOR}, \text{H}^+/\text{ROOH}$	+770–1440
$\text{O}_2^{\bullet-}, 2\text{H}^+/\text{H}_2\text{O}_2$	+940
$\alpha\text{-TO}^\bullet, \text{H}^+(\text{Vit. E radical})/\alpha\text{-TOH (Vit. E)}$	+500
$\text{H}_2\text{O}_2, \text{H}^+/\text{H}_2\text{O}, \bullet\text{OH}$	+320
$\text{Asc}^{\bullet-}, \text{H}^+ (\text{Ascorbyl rad.})/\text{AscH}^- (\text{Ascorbate})$	+282
$\text{GSSG}/2\text{GSH}$	–248

convenient to use thermodynamic properties to predict a hierarchy for free radical reactions. The most important thermodynamic quantity to characterize the course of a free radical reaction is the half-cell reduction potential [11]. For example, the one-electron reduction of a compound “A” is related to the half-cell reduction potential of the couple:



The overall chemical reaction for an oxidation-reduction couple can be described by the following reaction equation

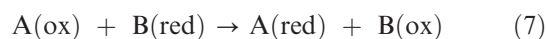
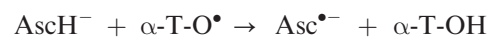


Table 2.1 summarizes half-cell reduction potentials of selected couples. The values are listed from highly oxidizing (at the top of Table 2.1) to highly reducing (at the bottom of Table 2.1). Any oxidized species is capable of taking an electron (hydrogen) from any reduced species occurring below it in Table 2.1, or, conversely, each reduced species is able to donate an electron (hydrogen) to any oxidized species above it in Table 2.1 [11].

As an example of these equations we refer here to a very important reaction that takes place in biological systems, namely, the regeneration of vitamin E by vitamin C. Based on the half-cell reduction potential values of the α -tocopherol radical ($\alpha\text{-T-O}^\bullet$)/ α -tocopherol ($\alpha\text{-T-OH}$, vitamin E) couple and an ascorbate radical anion ($\text{Asc}^{\bullet-}$)/ascorbate monoanion (AscH^- , vitamin C) couple it is clear that the ascorbate monoanion can react with the tocopherol radical to regenerate vitamin E:



2.3 OXIDATIVE STRESS AND REDOX ENVIRONMENT OF A CELL

Similar to the process of regulation of pH, biological systems tightly regulate the redox state of a cell [9, 11].

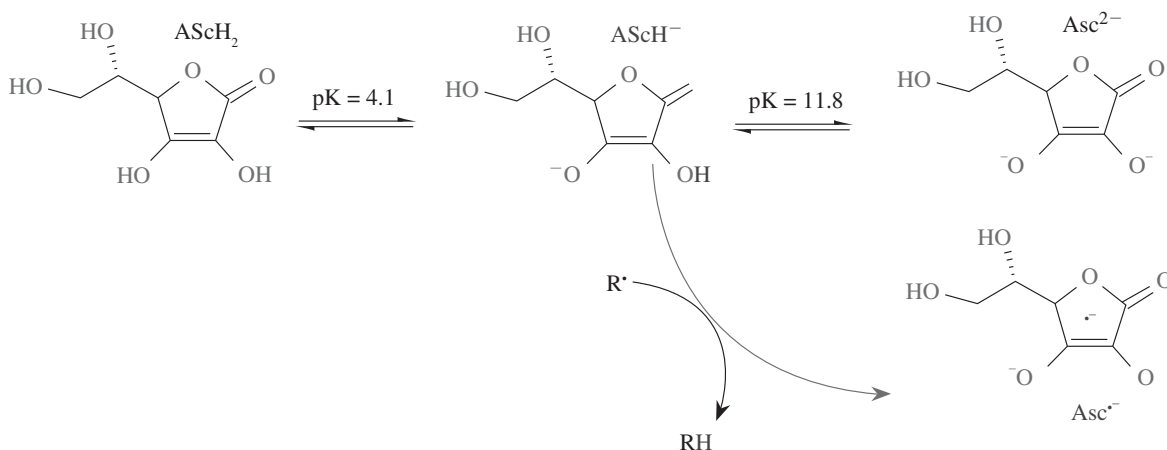


Fig. 2.1 Forms of vitamin C at various pH and its reaction with free radicals.

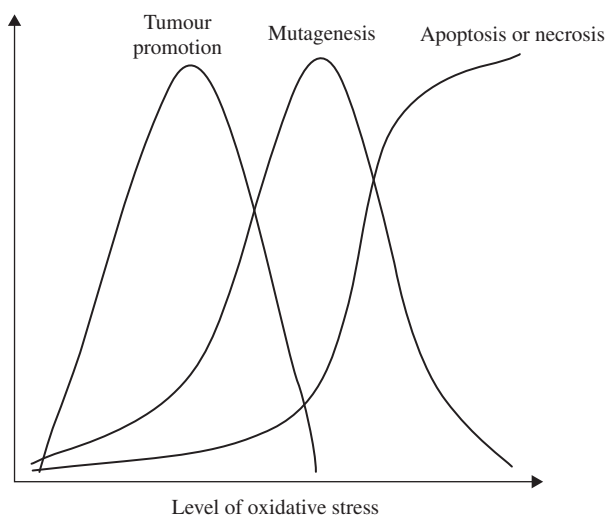


Fig. 2.2 Dose-dependent effect of relationship between level of oxidative stress and the tumor promotion process, the process of mutagenesis, and the process of apoptosis/necrosis.

The redox state of cells is kept in a narrow range and is given by the total “number of electrons” contained in various cellular constituents. The redox state of cells is maintained by the equilibrium of redox pairs, for example, $\text{Asc}^{\bullet-}/\text{AscH}^-$ (Asc = ascorbate), GSSG/GSH , and other couples. The tripeptide glutathione couple is one of the major cellular redox buffers [12]. Thioredoxin is an oxidoreductase enzyme and also participates in the maintenance of redox homeostasis [13]. In the course of the reduction of disulfide bonds by glutathione, this tripeptide is converted to its oxidized form, glutathione disulfide (GSSG). Conditions such as increased oxidative stress lead to increased pools of GSSG, which in turn lead to increased content of protein mixed disulfides. Proteins containing critical thiols that function as

receptors in cell signaling pathways can thus possess altered properties [14]. This points to the fact that GSSG appears as a nonspecific signaling molecule. From this it follows that maintenance of high ratios of reduced to oxidized forms of glutathione and thioredoxin substantiated by the action of GSH reductase and flavoenzyme thioredoxin reductase, respectively, is of key importance in the mechanism of redox homeostasis.

In addition to the above-described redox buffering systems, there are low-molecular-weight antioxidants equilibrating redox homeostasis, of which one of the most important is ascorbic acid [15]. Ascorbate is a diacid containing two hydroxyl groups that can undergo ionization. Antioxidant activity of ascorbate is realized through the ascorbate anion form (AscH^-), the most abundant form of ascorbate under physiological conditions. AscH^- is a donor antioxidant that reacts with free radicals forming the semidehydro-ascorbate radical anion ($\text{Asc}^{\bullet-}$) (Fig. 2.1). Thus exchange of electrons (and hydrogen atoms) between molecules of antioxidants and radicals determines the overall redox capacity of biological systems.

Changes in the redox environment of a cell are tightly linked with the cell cycle. A reducing environment is typical for cell proliferation [16]. Minor shifts in redox state toward a slightly oxidizing state are typical for cell differentiation. A more oxidizing environment of a cell is typical for apoptosis and necrosis (Fig. 2.2). This conclusion has been achieved using hydrogen peroxide, another oxidizing molecule significantly affecting the redox state of a cell. Experiments using cell lines showed that while low concentrations of hydrogen peroxide (up to $35\mu\text{M}$) induce apoptosis, necrosis is induced by high concentrations of oxidizing substances (more than $100\mu\text{M}$). A molecule of hydrogen peroxide is an uncharged species and can enter the cells by crossing the

biological membranes, a process that allows fluctuation of the redox environment of a cell.

2.4 ROS, SIGNAL TRANSDUCTION, AND HUMAN DISEASE

Signal transduction mechanisms enable induction of various biological processes, including cell growth, gene expression, muscle contraction, and others [17]. Proper functioning of such processes requires the presence of ROS and RNS acting as signaling molecules at various levels of the signal transduction process. Cytokines, growth factors, and hormones including, for example, interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α stimulate formation of low levels of ROS/RNS. ROS production is a basis for constitution of the “physiological oxidative burst,” necessary for the activation of various signaling pathways important for the maintenance of various physiological processes [9]. Redox-responsive signaling pathways control many physiological processes, for example, cell adhesion, immune response, NO production, sensing of oxygen concentration, regulation of vascular tone, and others.

Redox dysregulation substantiated by enhanced oxidative stress is a common denominator of various pathological conditions including cancer, neurological disorders, cardiovascular disease, metabolic disease, and aging [18]. Disease can be divided into two categories on the basis of origin: (i) The first group is represented by “mitochondrial disease” characterized by dysfunctional mitochondria demonstrated by the enhanced level of oxidative stress. The most typical disorders of this origin are cancer and diabetes mellitus. (ii) The second group of diseases are typical “inflammatory and oxidative” conditions. In addition, an enhanced activity of NAD(P)H oxidase leading to excess of ROS has been noted. This group of diseases is characterized by the enhanced levels of lipid peroxidation process, protein oxidation, and DNA damage caused by free radicals. The most typical diseases of this group are ischemic injury and atherosclerosis.

2.4.1 Cancer

As discussed above, low and transient concentrations of oxygen species participate in the process of cell proliferation [19]. On the other hand, high concentrations of oxygen species cause cell death and necrosis. ROS, redox-active, and redox-inactive metals interact with sulfhydryl groups of cysteine residues exposed on protein surfaces. The structural changes that occur at the active site of proteins trigger activation of several signaling cascades [19]. These include MAPK- and

PI3-kinase-dependent signaling pathways and growth factor kinases—all of which in turn lead to activation of redox-dependent transcription factors AP-1, p53, HIF-1, NF- κ B, and others (Fig. 2.3).

2.4.1.1 *Dysregulation of Cellular Signaling in Cancer*

Protein tyrosine phosphorylation plays a major role in various cellular processes such as proliferation, differentiation, and survival/apoptosis. The overall process of phosphorylation is driven by two antagonistic chemical reactions catalyzed by the protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) [20].

Ligands that trigger receptor tyrosine kinases involve insulin, insulin-like growth factor, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor, and others. Growth factors/growth factor receptors play a significant role in development, wound healing, and growth [9, 19].

Many cancers are tightly linked with the disruption of proper functioning of growth factors. Several lines of evidence have documented the influence of carcinogenic metals such as nickel, arsenic, and beryllium on growth factor receptors. These involve EGF receptor (EGFR), PDGF receptor (PDGFR), and VEGF receptors (VEGFR) [21].

Nickel compounds have been found to induce malignant tumors after intramuscular administration [22]. Overexpressed EGFRs have been detected in cancers of the urinary tract and lung cancers after exposure to increased concentration of nickel. The most profound carcinogenic effect of nickel has been reported for insoluble nickel compounds. Inhalation of nickel dust has also been associated with the development of cancers. The carcinogenic action of nickel is accomplished either by indirect damage through inflammation or directly by oxidative DNA damage via catalytic decomposition of hydrogen peroxide (Fenton reaction) forming reactive hydroxyl radicals [5].

Disruption of VEGF/VEGFR and EGF/EGFR pathways has been observed after arsenic exposure [23]. EGFR pathway activation has been shown to be activated by arsenic with the possible consequence of lung cancer [24]. Despite the various responses in patients suffering from non-small-cell lung cancers, a therapeutic approach based on the application of oral EGFR tyrosine kinase inhibitors (TKIs) appears to be a certain hope.

Overexpressed PDGF after exposure to arsenic has been described in lung, prostate, and ovarian cancers [25]. With the discovery of the new PDGF family members PDGF-C and PDGF-D, it has been shown that they play a role in renal disease, brain tumors (glioblastoma multiforme), and organ fibrosis.

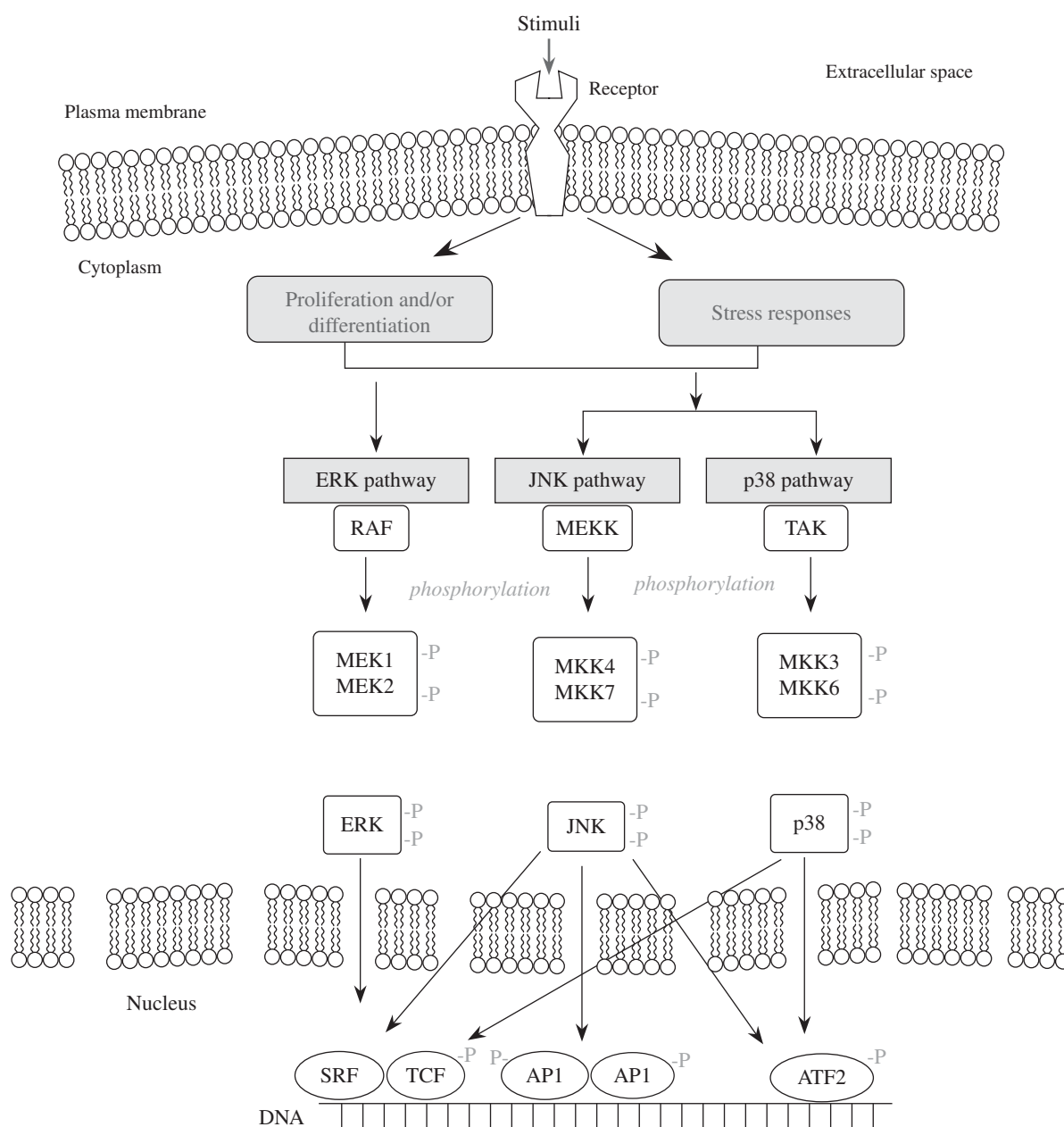


Fig. 2.3 Extracellular stimuli activate the MAPK pathways. Once MAPK kinase kinases [RAF, MEKK (MAPK/ERK kinase kinase) and TAK (TGF β -activated kinase)] are activated, they phosphorylate MAPKKs on two serine residues. MAPKKs in turn phosphorylate the MAPKs ERK (extracellular-signal-regulated kinase), JNK (JUN N-terminal kinase), and p38 on both threonine and tyrosine residues. Activated MAPKs can translocate to the nucleus to phosphorylate a number of transcription factors such as activator protein 1 (AP1) and activating transcription factor 2 (ATF2), thereby altering gene transcription. SRF (serum response factor); TCF (ternary complex factor).

Nonreceptor protein kinases (PTKs) that belong to the Src family and Janus kinase (JAK “just another kinase”) are also a target of ROS and RNS [26]. Hydrogen peroxide and superoxide radical anions have been documented to induce phosphorylation of several non-receptor protein kinases (PTKs) in various cell types, lymphocytes (T and B), and myeloid cells. PTKs belong to the Src family (Src

kinases) and Janus kinase (JAK “just another kinase”). Src activated by arsenic(III), UV radiation, and chromium (III) triggers MAPK signaling pathways. Overexpressed Src has been documented in cancers of colon, breast, pancreas, and bladder.

ROS interact with protein tyrosine phosphatases (PTP) and regulate phosphorylation of many important

signaling molecules, for example, of the MAP kinase family [27]. PTPs are a direct target of ROS and have been involved in regulation of oncogenic transformation, cell growth, and differentiation.

There are four main groups of MAPK family in mammalian cells, and they are serine/threonine kinases [28]. These kinases are known to phosphorylate hydroxy groups of serine or threonine. They are regulated by the various redox-active/-inactive metals, including cobalt, chromium, and nickel (redox active) and cadmium and arsenic (redox inactive).

The most significant effect of metals on signaling pathways has been observed in the mitogen-activated protein (MAP) kinase/AP-1 and NF- κ B signaling pathways, which are significantly affected by the effect of various stressors involving redox and nonredox metals and free radicals [5, 29].

Radiation, alcohol, benzpyrene, tetracyclic diterpenoids, asbestos, and other carcinogens represent external stress factors that activate NF- κ B [30]. The exact mechanism of such activation is not yet clear; however, its role in cell growth, differentiation, and inflammation has been thoroughly described. ROS can be considered as second messengers involved in activation of NF- κ B through IL-1 and TNF. Activated NF- κ B has been reported for colon, breast, and pancreas cells [29]. The involvement of ROS and metals in the activation of NF- κ B has been confirmed by studies utilizing antioxidants [31]. These studies suggested that thiols, polyphenols, carotenoids, vitamin E, L-cysteine, and other antioxidants can block activation of NF- κ B by various stressors.

p53 is considered as one of the transcription factors sensitive to oxidative stress that has the ability to halt the cell cycle or initiate apoptosis and thus protect cells from tumorigenesis [32]. p53 is known to induce the expression of p85 (a regulator of PI3K), which may function as a signaling molecule during p53-triggered apoptosis. p53 is activated by various stress factors, including hypoxia, gamma radiation, UV radiation, and others.

The effect of metals on p53 has been reported; however, the mechanisms are not fully understood [33]. In the case of the effect of arsenic on p53 several controversial reports have appeared in the literature spanning from no effect of arsenic on p53 to an induced p53 phosphorylation. In patients with arsenic-related skin disease an overexpression of p53 gene has been reported. Zinc is very important in the binding of p53 to DNA; thus isostructural metals capable of replacing zinc in its binding sites may affect p53 functioning [34].

Mutations in p53 have been noted on exposure to nitric oxide [35]. A close association between iNOS expression and mutations in p53 has been detected in stomach, brain, and breast cancers. Nitric oxide and derivatives of nitric oxide may cause mutations in

cancer-related genes and thus act as initiators/promoters of human carcinogenesis.

Metals can increase the intracellular levels of calcium, which activates the Ca^{2+} /calmodulin-dependent serine phosphatase calcineurin that in turn activates nuclear transcription factor NFAT [36]. In total there are five NFAT proteins evolutionarily related to Rel/NF- κ B, of which four are calcium-dependent. Redox-active metals iron, nickel, and vanadium exhibit the ability to activate NFAT. Vanadium has been shown to activate NFAT not only by a calcium-dependent pathway but also via formation of hydrogen peroxide.

HIF-1 regulates the expression of many cancer-related genes including VEGF [37]. VEGF plays an important role in tumor progression as well as angiogenesis and has been found to be expressed in many types of cancer. Similar to the previous factors, HIF-1 is activated mainly by hydrogen peroxide and carcinogenic metals, most profoundly by nickel [38]. In addition, HIF-1 very sensitively reflects oxygen homeostasis and hypoxia. A possible replacement of iron by nickel in the oxygen carrier hybrid hemoglobin leads to a steady-state hypoxia, thus activating HIF-1 via an oxygen-sensitive pathway.

HIF-1 has been described to participate in the glycolysis pathway and glucose transport [5]. In this connection studies have been done employing organic vanadium complexes, which have been documented to emulate actions of insulin via expression of HIF-1.

2.4.1.2 Redox Environment of a Cell and Mechanism of Carcinogenesis

There exist several theories explaining the mechanism of carcinogenesis [39]. One of the key theories is based on the disruption of an equilibrium between cell proliferation and cell death. Apoptosis is a normal physiological process that consists of the programmed mechanism of cell suicide [40]. A pivotal role in this process is played by the protein p53, and it is documented that more than half of cancer cases show defects in up- and downregulation of p53 expression. Uncontrolled apoptosis can destroy healthy cells; thus the delicate equilibrium between proapoptotic and anti-apoptotic regulation must be maintained. In view of this, cancer can be considered as a disturbed equilibrium between cell proliferation and cell death shifted toward cell proliferation.

A three-stage "initiation-promotion-progression" model of carcinogenesis involves changes in the redox environment of a cell reflecting the concerted action of ROS, RNS, and antioxidants [29, 39]. Initiation involves DNA mutations (e.g., 8-OH-Gua being the most studied) caused by oxidative DNA damage that occurs through the attack of free radicals and redox metals [41]. The process of initiation further passes through the release of calcium from intracellular calcium

stores. The stage of promotion is still a reversible and dose-dependent process that depends on the intensity of tumor promoters. Tumor promoters have strong inhibitory effects on the cellular pool of antioxidants. Progression is irreversible, and the final stage of the process of cancer development is characterized by the accumulation of genetic damage and genetic instability. During this stage the process of cell transformation from benign to malignant occurs.

The redox environment of a cell is tightly linked with all three stages of carcinogenesis [42]. Low to moderate levels of oxidative stress can stimulate cell division in the stage of tumor promotion. On the other hand, moderate to high levels of oxidative stress are cytotoxic for the cell, halting the proliferation by triggering apoptosis. Very high levels of oxidative stress induce necrosis in cells. Thus fine-tuning of the redox environment of a cell by redox-active compounds appears to be a way of affecting the cell cycle with the intent that the oncogenic process may be suppressed.

2.4.1.3 Cancer and Antioxidants Antioxidant defense represents one of the mechanisms of maintaining redox homeostasis [43]. The most effective antioxidants acting in biological systems involve antioxidant enzymes, of which the most important are SOD, catalase, and glutathione peroxidase. The low-molecular-weight antioxidants are represented by vitamins C and E, glutathione, carotenoids, flavonoids, and others.

Chronic gastritis and gastric metaplasia are both precancerous lesions, and they have been found in individuals with decreased serum levels of ascorbic acid [44]. This is in line with the epidemiological studies exploring the positive effect of ascorbic acid in reducing the incidence of stomach cancer. Similar findings have also been found in cancers of the lung and colon and rectum.

Several concerns have been raised over a possible prooxidant effect of ascorbic acid in the presence of redox metals such as iron. It has been claimed that vitamin C and iron can react to form damaging hydroxyl radicals [45]. However, these studies were performed under nonphysiological *in vitro* conditions. Under physiological conditions vitamin C acts as an antioxidant that is also capable of regenerating vitamin E from its radical form, α -tocopherol radical [46]. Vitamin C has been reported to regulate AP-1 complex. Ascorbate significantly (over 50%) inhibits JNK/AP-1 signaling pathways in UV-B irradiated cells.

Vitamin E in combination with vitamin C reduces the incidence of colorectal cancer by triggered apoptosis of cancer cells by inducing the powerful p21/WAF1/CIP1 belonging to the class of protein kinase inhibitors [47].

Glutathione is a very powerful cellular antioxidant regulating redox signaling by alterations in the level of

GSH and the ratio of GSSG (oxidized GSH) and GSH [48]. GSH activates various transcription factors involving NF- κ B and AP-1. GSH protects cells from apoptosis; thus the effectiveness of various anticancer drugs must be maintained or even enhanced by coadministered GSH-depleting agents. GSH-depleting agents are often used in the form of transition metal complexes.

Lycopene possesses antiproliferative effects on various cancer lines by inhibiting the cell cycle [49]. Regulated transcription factors, including inhibition of AP-1 and reduced induction of insulin-like growth factor I have been reported for prostate, lung, and breast cancers [3]. Beta-carotene has been shown to enhance proapoptotic effect in colon cell lines via a redox-driven mechanism of increased formation of ROS and GSSG-to-GSH ratio interconnected with enhanced NF- κ B activity. However, carotenoids, similar to other antioxidants, may behave under certain conditions as prooxidants [50]. To ascertain the effect of carotenoid supplements on human health, a long-term beta-carotene prevention trial was conducted by the National Institute of Health in Finland (ATBC trial) [51]. In this clinical trial supplemental beta-carotene (20 mg/day) was administered to 29,133 50- to 69-year-old male smokers in Finland for 5 to 8 years. The results were intriguing. It has been reported that men who took beta-carotene had an 18% increased incidence of lung cancers, which contributed to an 8% increased overall mortality! These findings suggest that carotenoids may elicit a prooxidant effect. Thus the antioxidant behavior of antioxidants depends not only on concentration of the antioxidant but also on the site of its action. In the case of beta-carotene, the oxygen-rich environment in lungs triggered the formation of car-OO \cdot radicals, which exhibited a significant harmful prooxidant effect.

Polyphenols represent another important class of compounds with antioxidant and chelating properties [52]. Their antioxidant capacity has a beneficial effect on human health. Flavonoids can prevent cancer, cardiovascular disease, and other pathological disorders. Increased flavonoid intake, mainly quercetin, has been associated with reduced incidence of lung, stomach, and pancreatic cancer.

2.4.2 Cardiovascular Disease

Oxidative stress contributes to the development of cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiac hypertrophy, and congestive heart failure [53].

Oxidative stress associated with enhanced formation of ROS and RNS has been linked to various cardiovascular disorders such as ischemic heart disease, hypertension, atherosclerosis, and heart failure [54]. Oxidative

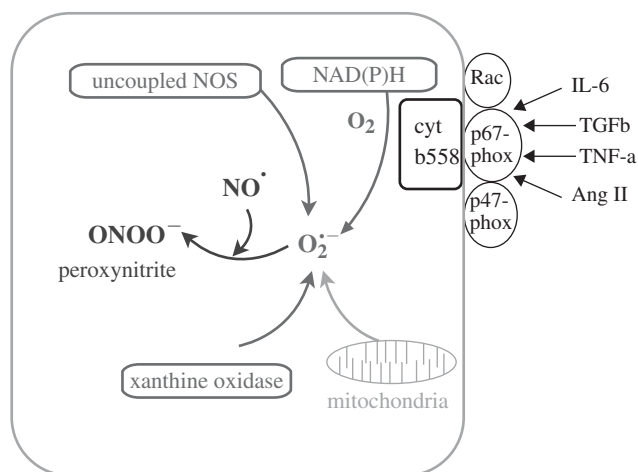


Fig. 2.4 Pathways of ROS and RNS generation in cardiovascular system.

stress in cardiac and cardiovascular systems has common denominators such as disruption of mitochondrial oxidative phosphorylation, activation of the xanthine dehydrogenase/xanthine oxidase system, uncoupled synthesis of nitric oxide, and activation of NAD(P)H by various activators (Fig. 2.4). The common feature for all these events is uncontrolled formation of superoxide anion radicals, which is the start of various deleterious radical reactions causing damage to biological tissues including the heart.

The most profound damage involves peroxidation and oxidation of thiol groups of biomolecules leading to changes in membrane fluidity and permeability, disruption of membrane lipids, and severe modification of various cellular proteins [55].

The studies dealing with the role of calcium in cardiovascular disease states are of key importance [56]. Heart mitochondria under conditions of oxidative stress show decreased membrane transport of calcium. Superoxide radical-incubated sarcolemma exhibited decrease in sarcolemmal ATP-dependent Ca^{2+} accumulation and calcium-stimulated ATPase activities. Radicals involving superoxide radicals, hydroxyl radicals, and nitric oxide all interact with sulphhydryl groups of ryanodine receptors, which in turn promote the release of calcium from the sarcoplasmic reticulum. Release of calcium results in the activation of kinases, such as protein kinase C (PKC), a member of the serine/threonine kinases. ROS-induced PKC activation has important functional consequences on downstream signaling pathways, namely, activation of MAPKs [57].

Several MAPK subfamilies have been identified in the mammalian cardiovascular system [30, 58]. The main MAPKs found in cardiac tissue are the extracellular signal-regulated kinases (ERKs), p38-MAPK, the

stress-activated/c-Jun NH₂-terminal kinases (SAPK/JNKs), and ERK5/big MAPK 1 (BMK1). The ERKs are activated by physical stress; SAPK/JNKs and p38-MAPK are activated by various cell stresses, such as metabolic stress, UV radiation, heat shock, cytokines, and ischemia (Fig. 2.3). Activation of MAPKs represents pathogenesis of various processes occurring in the heart, for example, heart failure and ischemic and reperfusion injury [59]. In this respect, pharmacological modulations of MAPK activity and their impact on gene targeting or expression is of key importance.

2.4.2.1 Mitochondrial Dysfunction and Cardiovascular Disease Mitochondrial oxidative phosphorylation occurs within mitochondrial inner membranes and generates mitochondrial ATP used predominantly in the cytosol [56]. To transport ATP to the outside of the mitochondrial matrix, the organelle uses the ADP/ATP transmembrane protein carrier adenine nucleotide translocase, which governs the exchange of newly synthesized ATP in the mitochondrial matrix for ADP in the transmembrane space. In addition to soluble enzymes and small organic molecules, the matrix contains ribosomes and mitochondrial DNA (mtDNA). Mutations to mtDNA are responsible for many human diseases [60]. Apart from neurological, endocrine, and renal diseases, cardiac and cardiometabolic disorders represent one of the major hallmarks of mtDNA mutations. Numerous experiments have confirmed that mtDNA is more prone to oxidative damage than nuclear DNA.

Mitochondrial production of superoxide radicals represents an evolutionary process by which cells regulate the concentration of various oxidants important in cell signaling pathways. However, mitochondrial formation of superoxide must be tightly controlled. Disruption of the mitochondrial balanced formation of superoxide radical leads to various diseases including the cardiovascular diseases. mtDNA mutations may lead to the enhanced formation of ROS and RNS, causing damage to mtDNA, which in turn triggers cardiovascular disorders [61]. To date more than several hundred mtDNA mutations have been reported, which can be divided into two major groups, point mutations and rearrangement mutations [56]. In fact, many mitochondrial diseases exhibit accumulation of mtDNA mutations and the progress of age-related decline in oxidative phosphorylation.

Production of mitochondrial ROS and RNS, mitochondrial antioxidants, and uncoupling protein activities, are all regulated by various physiological functions. Superoxide radicals can react with nitrogen oxide, forming peroxynitrite (ONOO^-). Peroxynitrite is a molecule with a damaging effect on various cellular components including DNA, proteins, and lipids. Mitochondrially generated superoxide radicals can be converted to

hydrogen peroxide by Mn-SOD [29]. Hydrogen peroxide is a signaling molecule that can be removed by catalase, forming water and oxygen. In the course of its production, hydrogen peroxide can be decomposed to damaging hydroxyl radicals by traces of transition metals such as iron(II) and copper(I).

Local concentrations of nitric oxide in mitochondria influence various process, such as superoxide radical formation and mitochondrial respiration. Relative concentrations of O_2 and NO thus influence the regulation of mitochondrial respiratory functions as well as concentrations of downstream reactive molecules, hydrogen peroxide and peroxynitrite [62]. An uncoupling protein (UCP) is a mitochondrial carrier catalyzing regulated electrophoretic proton transport across the inner mitochondrial membrane. Proton transport serves to reduce formation of oxidant molecules. The low expression levels of UCP are linked to suppressed proton transport and increased membrane potentials and increased formation of superoxide radicals [63]. The activation of UCPs is carried out by reactive alkenals, such as aldehydes (4-HNE) and other products of lipid peroxidation process.

The antioxidant pool is also an important factor in maintaining a physiological balance of mitochondrial oxidant molecules. Cytokines are a class of protein molecules that can directly or indirectly modulate the mitochondrial and cellular redox state. For example, PDGF mediates via mitogen-activated protein kinase 1 (MEK1) and ERK1/2 SOD2 transcription [64].

Mitochondria are exceptionally prone to damage by ROS and RNS. The most aggressive ROS and RNS causing damage to mitochondrial components are hydrogen peroxide and peroxynitrite, resulting in impaired mitochondrial protein synthesis and lowered redox state in vascular cells. This may lead, for example, to altered energy generation and redox signaling. It has been shown that prolonged ischemic injury in cardiac cells results in increased sensitivity of mitochondria to fluctuations in concentration of nitric oxide [65]. Protein synthesis inhibition in mitochondria has been documented by increased sensitivity to nitric oxide and induced apoptosis.

There is clear evidence pointing to an association between cardiovascular disorder incidence and mitochondrial impairments. Patients suffering from cardiovascular disease exhibited significantly increased abnormalities and damage to mtDNA and aorta compared with healthy subjects [66]. There are various molecular factors contributing to the increase of cardiovascular disease states. Among the most important is an elevated level of oxidative stress causing mitochondrial damage to the heart. The enhanced formation of free radicals participates in the increased mtDNA deletions

and peroxidation of lipids in mitochondria [67]. Protection against these deleterious effects and enhanced tolerance to ischemia are provided by the mitochondrial antioxidants preventing these harmful mechanisms. There is a direct correlation between suppressed activities of SOD2 and increased susceptibility to cardiovascular risk.

Mitochondrial antioxidants and UCP protect the cardiovascular system against oxidative stress and the effects of ischemia and reperfusion. Therefore a deficiency in antioxidants and UCP in the heart has been linked with the triggering of cardiovascular disease under in vivo conditions.

2.4.2.2 Atherosclerosis and Hypercholesterolemia

There is a clear correlation between DNA damage and atherosclerosis [68]. Various DNA adducts have been linked with the prerequisite of the development of atherosclerosis. As shown by ^{32}P -postlabeling experiments, atherosclerotic patients exhibited significantly increased levels of aromatic DNA adducts in the thoracic aorta compared with control subjects [69]. The most frequently observed DNA adduct is 8-OH-Gua, which was also detected in plaques of the human carotid arteries. In addition, signs of enhanced DNA repair mechanisms have been observed in the atherosclerotic tissues. In line with all these results is the observation of significantly damaged mtDNA in cardiac tissues. However, the question of whether this damage is the cause or consequence of the cardiovascular disease state remains open.

The increased iron pool observed in atherosclerotic plaques is a good indicator of the iron-catalyzed formation of hydroxyl radicals (e.g., via the Fenton reaction), which may contribute to the development of atherosclerosis [70]. Increased cholesterol level and the uptake of oxidized low-density cholesterol (oxLDL) have been found to participate in the development of atherosclerosis. OxLDL mediates the formation of the superoxide anion radical, triggering apoptosis of vascular wall and plaque formation. Formation of superoxide radicals, and their ability to oxidize nitric oxide by forming peroxynitrite, is known to initiate peroxidation of lipids and oxidation of lipoproteins. These processes play a key role in the development of atherosclerosis.

Cholesterol administration in laboratory animals resulted in impaired mitochondrial energetic functions [71]. The activity of mitochondrial dehydrogenases was also impaired. Hypercholesterolemia leads to increased DNA damage. This has been substantiated by the observed 8-OH-Gua immunoreactivity and DNA strand breaks in atherosclerotic plaques in rabbits fed a cholesterol-rich diet for 6 months. The level of DNA strand breaks returned back to normal within a

month. However, reduction of 8-OH-Gua required between 3 and 6 months. While a high-fat diet reduced expression of genes involved in synthesis of antioxidant enzymes such as SOD and GPX, expression of genes responsible for the synthesis of stress proteins (Hsp 70) increased [72]. Intake of antioxidant supplements simultaneously with a high-fat diet reduced the deteriorating effect of cholesterol.

2.5 DIABETES

The majority of diabetes patients are non-insulin-dependent (type 2 diabetes), and about 10 of all patients are insulin-dependent (type 1 diabetes). The development of diabetes has been linked with the presence of oxidative stress substantiated by the formation of superoxide radicals and peroxidation of lipids, which in turn lead to the formation of isoprostanes, malondialdehyde, 3-nitrotyrosine levels, and DNA damage. DNA damage has been documented by the increased presence of oxidized DNA bases (one of the most abundant being 8-OH-Gua) in urine samples from diabetic individuals [73].

Under physiological conditions, complex I and ubiquinone-complex III in the mitochondrial membrane are major sources of electrons for the formation of ROS. However, under pathological conditions of diabetes mellitus, the primary site of superoxide radical formation becomes complex II [74]. This finding was revealed after application of the complex II inhibitor 2-thenoyl-trifluoroacetone, which led to a decrease in ROS formation after treatment of various cell lines with high concentrations of glucose [75]. Formation of superoxide radicals in diabetic patients in mitochondria further increases the proton gradient across the inner mitochondrial membrane due to the overproduction of electron donors, for example, NADH. Overexpression of mitochondrial SOD2 counteracts the effect of superoxide radical, which in turn suppresses the activation of IL-1 β /TNF- α /IFN- γ of NF- κ B and induction of iNOS in insulin-producing cells. Conversely, suppression of SOD2 results in greater activation of NF- κ B. These experimental findings suggest that mitochondrially derived ROS play a key role in the activation of the cytokine-sensitive transcription factor NF- κ B.

NADPH oxidases are another major source of glucose-induced formation of free radicals and are considered as major mediators of diabetic complications [76]. Glucose-induced formation of free radicals by NADPH can be suppressed by the application of PKC inhibitors, pointing to the importance of this family of kinases in the regulation of hyperglycemia.

In addition to ROS, RNS has been implicated in the etiology of diabetes. NO \bullet forms with O $_2^{\bullet-}$ harmful

peroxynitrite (ONOO $^-$), which reacts with the zinc cluster of NOS, leading to its uncoupling. Thus ONOO $^-$ not only depletes NO \bullet but, more importantly, causes damage to NOS and thus suppresses the formation of NO \bullet .

Xanthine oxidase (XO) is another source of free radicals under diabetic conditions [77]. Allopurinol is an effective XO inhibitor and has been shown to reduce the concentration of oxidized lipids in plasma and to positively influence the blood pressure in type 2 diabetes patients.

Formation of ROS and RNS by the above-described sources depletes the antioxidant enzymes and low-molecular-weight antioxidants. Vitamin E supplementation in diabetic patients had a protective effect, mainly with respect to the level of lipid peroxidation [78]. The role of vitamin C, mainly at the plasma level in diabetic complications, has been studied. However, the results are not conclusive. Glutathione peroxidase (GPX) protects the organism from oxidative damage, and its relevance to diabetic complications has been investigated [79]. Hyperglycemia has been shown to affect the expression of GPX; however, the extent of the inhibition of expression and how it affects the cells is unclear.

The consequences of increased oxidative stress under diabetic conditions can sensitively be monitored with the use of appropriately selected biomarkers [80]. The most relevant include urinary and plasma levels of malondialdehyde and isoprostanes (nonenzymatic products of the oxidation of arachidonic acid). The role of 4-hydroxynonenal has not been proven. Modifications of side chain protein groups are referred as advanced glycation end-products (AGEs). AGEs were found in many tissues of various origin in rats and non-insulin-dependent humans [81].

2.6 NEUROLOGICAL DISORDERS

Alzheimer disease (AD) is a neurological disorder characterized by the presence of amyloid plaques and neurofibrillary tangles in the brain [82]. The main constituent of amyloid plaques is a 39- to 42-residue peptide, amyloid- β protein (A β). Besides this, the major pathological feature of AD is the presence of an aberrant form of tau protein accumulated in the neurons in the form of neurofibrillary tangles.

The major neuropathological hallmark of Parkinson disease (PD) is the occurrence of intracellular inclusions called Lewy bodies that are located within the cytoplasm of neurons and consist of granular materials and filaments [83]. Lewy bodies show dense protein central cores with a rim of radiating filaments (7–20 nm in diameter).

2.6.1 Alzheimer Disease

Recent advanced scattering spectrometry experiments have shown significantly increased contents of metals within the amyloid plaques in AD brain with respect to surrounding tissue [84, 85]. The amyloid plaques have been found to contain significant amounts of A β organized into amyloid fibrils. Two major peptide fragments are formed, A β (1-42) and A β (1-40), displaying a different neurotoxicity that correlates with a marked difference in aggregation behavior [85]. While synthetic A β (1-40)s primarily exist as a monomer/dimer mixture, A β (1-42) contains a transient low-order potentially toxic oligomeric species. An increasing body of evidence suggests that various oligomeric A β s of different sizes are involved in the development of AD.

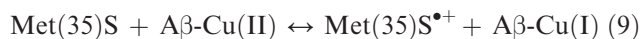
While the majority of papers dealing with the origin of AD have considered the amyloid cascade hypothesis as the “null hypothesis,” there is an alternative view proposing that amyloid- β is not a primary cause, but more probably a secondary event of the disease [86].

Without regard to such views, there is clear evidence that accumulated amyloid- β is able, under pathological conditions, to generate free radicals. Thus the pathology of AD is directly linked with the enhanced occurrence of oxidative stress of the brain [87]. The underlying factor of oxidative stress in the brain is the disrupted homeostasis of redox metals iron and copper and redox-inert metal zinc.

Copper(II) is abnormally elevated in amyloid plaques of AD brain and binds to A β through His13, His14, His6, and Tyr10 amino acid residues [88]. Besides copper (II), A β also binds iron(III) and zinc(II). Both metals are elevated in the amyloid plaques of subjects with AD. The neurotoxicity of A β is linked with its ability to reduce Cu(II) \rightarrow Cu(I) and form hydrogen peroxide. The neurotoxicity of A β can be attenuated by the administration of free radical scavengers and various antioxidants including vitamin E.

The redox-inert metal zinc has a special role in AD. While the molecular mechanism of the action of zinc in AD is largely unknown, its preventive effects against A β toxicity in micromolar concentrations have been well documented. On the other hand, enhanced copper and iron-induced oxidative and nitrosative stress causes the release of zinc from vesicular pools, which may have serious neurotoxic consequences. Thus, under normal physiological conditions, there is a sensitive balance among zinc, copper, iron, and A β metabolism. Deposition of redox-active copper and iron induces an increase in oxidative stress that in turn may perturb the subtle metal ion balance substantiated by the uncontrolled zinc elevation from vesicular pools and, possibly, amyloid deposition.

Methionine35 (Met35) is significantly abundant in AD brain, which is in agreement with the high susceptibility of the sulfur atom of methionine to oxidation [89]. A recently presented model involves an oxidation reaction between C-terminal methionine (Met35) with N-terminally complexed Cu(II) according to the reaction

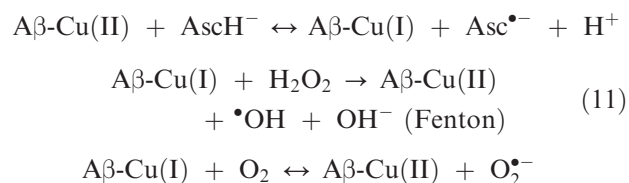


forming the sulfide radical of methionine35 [Met(35)S $^{\bullet+}$] and cuprous ions. The process of reduction of copper(II) is achieved via one-electron oxidation of methionine(35) forming the methionine radical cation MetS $^{\bullet+}$, which plays a role in free radical formation and the neurotoxicity of A β . Met35 is the most vulnerable residue in A β to oxidation and may react with various radicals including superoxide radical anion [89, 90].



thus forming the Met-sulfoxide product (MetSO), which has been detected in AD senile plaques. Oxidation by the Cu $^{2+}$ -A β complex involves cell components such as fatty acids and cholesterol, leading to the formation of various markers of peroxidation process, the most neurotoxic being malondialdehyde (MDA), peroxynitrite, heme oxygenase (HO-1), and AGEs. Reduced copper (cuprous ions) can participate (via the Fenton reaction) in the catalytic decomposition of hydrogen peroxide, thus forming the reactive hydroxyl radical, which in turn is involved in the lipid peroxidation process. AGEs further activate proinflammatory cytokines, for example, IL-6. In addition, tyrosine residues can be a target of free radical attack, as documented by the observed accumulation of dityrosine and 2-nitrotyrosine in AD brain.

In vitro experiments employing neurotoxic forms of A β , A β (1-42) and A β (1-40), have been shown to stimulate copper-mediated oxidation of ascorbate (AscH $^-$) and formation of hydroxyl radicals. The following mechanisms have been proposed [87]



From the proposed mechanism it follows that copper (II) and ascorbate in the presence of hydrogen peroxide and oxygen (both of which are present in relatively high content in brain cells) leads to the formation of free radicals with damaging effects [5 and references therein].

We note that the above-described model is based on *in vitro* experiments and many other clinical trials employing high doses of vitamin C have disproved such conclusions. Based on the findings under *in vivo* conditions, it has been concluded that even high doses of vitamin C are not harmful to the organism (if not beneficial) and vitamin C does not act as a prooxidant [46]. In addition, vitamin C is able to regenerate vitamin E from its radical form (α -tocopheroxyl radical) back to α -tocopherol. Such combinations of preventive and chain-breaking antioxidants such as vitamin C and vitamin E could protect brain lipoproteins against oxidative stress.

An epidemiological trial of the use of vitamin E (2000 IU/day, 2 years) in patients with moderate AD has shown slowed functional deterioration (–53%) [91]. In addition, a combined supplemental intake of vitamins E and C was found to reduce prevalence (–78%) and incidence (–64%) of AD in elderly people [91]. In light of these results, vitamin E appears to act in concerted action with other antioxidants, predominantly with vitamin C, and thus provide protection of A β against oxidative damage.

2.6.2 Parkinson Disease

Parkinson disease (PD) is a progressive degenerative disorder of the central nervous system that affects motor skills and functions [92]. The majority of PD cases are sporadic (90–95%). Familial cases account for 5–10% of PD. Some studies have concluded that familial and sporadic PD patients display similar clinical features.

The main feature of PD is the degeneration of dopaminergic neurons containing and synthesizing the neurotransmitter and neurohormone dopamine in the substantia nigra pars compacta (SNc).

The most typical pathological feature of PD is the presence of intracellular inclusions called Lewy bodies that consist of aggregates of the presynaptic soluble protein called α -synuclein [93]. α -Synuclein is mainly a neuronal protein localized in neuronal mitochondria; however, it is also present in glial cells.

A relatively high level of oxidative stress in the SNc with respect to other brain regions has been found in postmortem studies of the brain of PD patients. The earliest signs of PD development are characterized by the rapid depletion of the antioxidant glutathione in the substantia nigra [94]. The loss of glutathione may have an effect on energy production in the mitochondria, most probably linked with the decline in the activity of mitochondrial complex I. α -Synuclein is localized in the inner membrane of mitochondria, and its inhibitory effect on mitochondrial complex I activity is dose dependent. Apart from increased markers of oxidative

stress in the SNc, other factors, involving inflammation and the toxic action of NO \cdot , may play a role in the development of PD [95, 96]. The role of trace metals has also been studied, showing increased iron levels in the PD midbrain, catalyzing the process of neurodegeneration [94]. The harmful effect of iron can be suppressed by the application of the iron chelator clioquinol [97]. Chelated iron in the form of the Fe-clioquinol complex does not participate in the formation of free radicals and prevents degeneration of dopaminergic midbrain neurons. The major features indicating the presence of oxidative stress in PD are lipid peroxidation markers, including 4-hydroxy-trans-2-nonenal (HNE), 4-oxo-trans-2-nonenal (4-ONE), 4-oxo-trans-2-hexenal, acrolein, and malondialdehyde. In addition, defects in protein clearance and toxic action of nitric oxide are all factors contributing to the development of PD.

In addition to α -synuclein, four other genes have conclusively been linked to dominant (LRRK2) and autosomal recessive (parkin, PINK-1, DJ-1) parkinsonism [98, 99]. Mutations in the LRRK2 gene are the most common cause of genetic PD mutations in PINK1. The PINK1 gene produces a protein called PTEN induced putative kinase 1, and mutations in PINK1 are the second most frequently cause of autosomal recessive parkinsonism. Mutations in the parkin gene induce a loss of parkin function, leading to the hypothesis that the accumulation of parkin substrates causes neurotoxicity and results in the death of dopaminergic neurons. Recent experiments revealed that the mutated protein Parkin is transported from the cytoplasm to damaged mitochondria and that this causes the breakdown of the mitochondria by processes acting within the cell.

DJ-1 is present mainly in the cytoplasm and less in the mitochondria and nucleus of dopaminergic cells [96]. Recently presented results suggest that various environmental toxins that may induce oxidative stress are linked with the role of DJ-1 [100]. Loss of DJ-1 leads to noteworthy susceptibility to the herbicide paraquat and the insecticide rotenone. This points to a possible role of DJ-1 in the protective action against environmental toxins inducing oxidative stress.

2.7 CONCLUSION

ROS and RNS are species possessing dual characters, acting under normal conditions as signaling molecules involved in many physiological functions of living systems and, conversely, under pathological conditions possibly inducing oxidative stress. Ironically, various ROS-mediated actions in fact protect cells against ROS-induced oxidative stress and reestablish or maintain “redox balance,” also termed “redox homeostasis.” The

dual character of ROS as pro- and antitumorigenic species is documented by the fact that on one hand they maintain the oncogenic phenotype of cancer cells but on the other hand they can also induce cellular senescence and apoptosis.

Overproduction of ROS resulting in oxidative stress is frequently achieved by excessive stimulation of NAD(P)H by cytokines, or by the mitochondrial electron transport chain and xanthine oxidase. The current problem in the quantification of the level of oxidative stress with respect to human disease and ageing is to determine the most reliable oxidative stress markers. Following this, the monitoring of healthy subjects over a few decades will be necessary to obtain reliable results.

Further effort is required to design effective redox-active agents interfering with the mechanism of ROS-induced apoptotic pathways. Detailed description of NO-driven redox-mediated signaling is anticipated to develop novel therapeutics for heart failure.

Antioxidant enzymes and low-molecular-weight antioxidants provide protection against deleterious effects of oxidative stress. Since redox-active and redox-inert metals directly or indirectly participate in the formation of damaging ROS and RNS, the design of dual-functioning antioxidants, possessing both metal-chelating and ROS/RNS-scavenging properties is anticipated.

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OXIDATIVE STRESS AND ITS BIOCHEMICAL CONSEQUENCES IN MITOCHONDRIAL DNA MUTATION-ASSOCIATED DISEASES: IMPLICATIONS OF REDOX THERAPY FOR MITOCHONDRIAL DISEASES

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3.1 INTRODUCTION

Mitochondrial diseases are a heterogeneous group of disorders characterized by mitochondrial dysfunction. Defects in any of the numerous mitochondrial pathways including electron transport chain and oxidative phosphorylation (OXPHOS) can be caused by mutations in either mitochondrial DNA (mtDNA), which has only 37 genes, or nuclear DNA (nDNA), which encodes most of the mitochondrial proteins and all other proteins required for mitochondrial metabolism [1]. Because most of the mitochondrial diseases involve tissues with high energy demand such as brain and skeletal muscle, respiratory chain disorders caused by mtDNA mutations deserve special attention. Human cells contain hundreds or thousands of mitochondria, and each mitochondrion contains approximately 2–10 copies of mtDNA, which are distributed randomly among the daughter cells at cell division. When a mutation occurs in the mitochondrial genome, it usually affects only a portion of mtDNA and thereby results in the coexistence of two populations of mtDNA (wild type and mutant) within a cell, tissue, or individual, and this phenomenon is termed heteroplasmy [2, 3]. Therefore, clinical manifestation of a pathogenic mtDNA mutation is determined in large part by the

relative proportion of normal and mutant mtDNAs in different affected tissues including muscle, blood, and hair follicles. Up until now, mutations in the mitochondrial genome have been mostly documented in a heterogeneous group of disorders in which the nervous system and the skeletal muscle are predominantly affected [4, 5].

The majority of mitochondrial diseases are maternally inherited because mtDNA is transmitted from the maternal lineage [6]. A mother carrying an mtDNA mutation can pass it to all her children (boys and girls), but only her daughters will transmit the mtDNA mutation to their children [7]. However, evidence has been obtained to show that there can be paternal inheritance of mtDNA, but such an event appears to be quite rare [8]. Identification of pathogenic mtDNA mutations were first reported in 1988 [9, 10], which provided the basis for modern molecular genetics and the classification of mitochondrial diseases. In the past two decades, more than 250 pathogenic mtDNA mutations have been detected in the affected tissues of patients with mitochondrial diseases [11]. Most of the affected individuals harbor mutations in the transfer RNA (tRNA) or protein-coding genes in mtDNA and display a cluster of clinical features that fall into discrete clinical syndromes. The notable syndromes include mitochondrial encephalomyopathy, lactic

acidosis and strokelike episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF), neurogenic muscle weakness, ataxia, retinitis pigmentosa (NARP), Leber's hereditary optic neuropathy (LHON), and Leigh syndrome. In addition, the deletion and duplication of mtDNA are associated with a wide spectrum of mitochondrial diseases including chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome (KSS), diabetes mellitus, deafness, and hypertrophic or dilated cardiomyopathy [12–14]. Specific mutations of mtDNA have been identified to cause or be associated with these mitochondrial diseases. In addition, some mtDNA mutations have also been documented in a number of genetic disorders and neurodegenerative diseases, including Parkinson disease (PD) [15], Alzheimer disease (AD) [16], amyotrophic lateral sclerosis (ALS) [17], and Wilson disease [18], and also as one of the contributory factors for aging [14, 19]. It was estimated that as many as 1 in 10,000 people have clinically manifesting mitochondrial disease and a further 1 in 6000 people are at risk [20]. Importantly, the correlation between the phenotype (clinical symptom) and genotype (mtDNA mutation) is rather poor for mitochondrial diseases [21]. In addition, the molecular mechanisms that underlie the complexity and diversity of the pathophysiology of mitochondrial diseases remain unclear.

3.2 MITOCHONDRIAL DNA MUTATION-ELICITED OXIDATIVE STRESS

Mitochondria are the powerhouse of human cells and are responsible for the supply of the majority of ATP by the OXPHOS machinery, which is located on the inner membranes of mitochondria. The structure of the mitochondrion is bounded by a double membrane that divides the mitochondrion into four distinct parts: the outer membrane, intermembrane space, inner membrane, and matrix. The mitochondrion has its own DNA molecules in the mitochondrial matrix. Human mtDNA is a 16,569-bp circular, double-stranded DNA molecule, which encodes 13 polypeptides that constitute the OXPHOS system as well as 2 ribosomal RNAs (rRNAs) and 22 tRNAs, which are essential for protein synthesis in mitochondria [22]. The mitochondrial electron transport chain and OXPHOS system comprise five multi-subunit enzyme complexes: complex I [nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase], complex II (succinate-ubiquinone oxidoreductase), complex III (ubiquinol-cytochrome *c* oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase) [22]. In addition, two mobile electron carriers, ubiquinone and cytochrome *c*, are also involved in the elaborated electron transport process.

3.2.1 Generation of Reactive Oxygen Species in the Electron Transport Chain

The electron transport chain of mitochondria is involved in a series of redox reactions. After the reducing equivalents are transferred to the respiratory chain from NADH via complex I or from reduced flavin adenine dinucleotide (FADH₂) via complex II, electrons pass down to complex III and IV via ubiquinone and cytochrome *c*, respectively, and finally reduce molecular oxygen (O₂) to form water (H₂O). This series of electron transfer reactions accompany the proton pumping from matrix to intermembrane space to establish an electrochemical H⁺ gradient, which is then used by complex V to drive ATP synthesis. Under normal physiological conditions, most of the tissue oxygen (over 90%) is consumed by mitochondria in human cells [23]. Although the electron transport process efficiently shuttles electrons to O₂, about 1–5% of the consumed O₂ is transformed to superoxide anions (O₂^{•−}) via “electron leak,” particularly at complex I (at the bound FMN on the matrix side) and complex III (at the ubiquinol oxidation side) [24]. The O₂^{•−} can be rapidly metabolized into hydrogen peroxide (H₂O₂) in mitochondria by manganese-dependent superoxide dismutase (Mn-SOD), and then H₂O₂ can be detoxified by catalase (CAT) or glutathione peroxidase (GPx) into H₂O. However, if O₂^{•−} or H₂O₂ is not promptly and efficiently removed, highly reactive peroxynitrite (ONOO[−]) or hydroxyl radical (HO[•]) could be produced in the presence of nitric oxide (NO) or Fe²⁺ and Cu⁺, respectively [24, 25]. It has been reported that the production of intracellular reactive oxygen species (ROS) is directly proportional to the rate of mitochondrial oxygen consumption of the mammalian cells [26]. Therefore, while the mtDNA mutations exist in affected tissue cells, the rate of mitochondrial ROS production would be significantly increased in the mutant cells, as expected.

3.2.2 Vicious Cycle of Mitochondrial Oxidative Stress

Since mitochondria are the major source of ROS and mtDNA is not protected by binding with histonelike proteins, human mtDNA is inflicted with more oxidative damage compared with nDNA in mammalian cells [22, 27]. In addition, the mutation rate of human mtDNA was reported to be about 20 times higher than that of nDNA because of the lack of an efficient DNA repair system in mitochondria [28]. The accumulation of mtDNA mutations in brain tissues is thought to lead to the neurodegenerative disease-associated decline of mitochondrial function [19, 29]. Indeed, defective mitochondrial respiratory enzymes have been frequently observed in the affected tissues of patients with mitochondrial diseases caused by mtDNA mutations (Table 3.1). Therefore,

TABLE 3.1 Clinical phenotype and mtDNA mutations with mitochondrial dysfunction in affected tissues or cells from patients with mitochondrial diseases

Patients	Biopsies	mtDNA mutation	RRF	OXPHOS dysfunction	Reference
MERRF	Muscle	A8344G (RNA ^{Lys})	+	Complexes I, III, and IV	57, 56
MERRF	Muscle	G611A (RNA ^{Phe})	+	Complex IV	66
MERRF	Cybrids	A8344G (RNA ^{Lys})	NA	O ₂ consumption rate (83% ↓)	67
MELAS	Muscle	A3243G (RNA ^{Leu})	+	Complexes I and IV	56, 68
MELAS	Muscle	A3243G (RNA ^{Leu})	+	Complex IV (95% ↓)	69
MELAS	Myoblasts	A3243G (RNA ^{Leu})	NA	Complexes I (80% ↓) and IV (37% ↓)	70
MELAS	Cybrids	A3243G (RNA ^{Leu})	NA	O ₂ consumption rate (55% ↓)	67
LHON	Muscle	G11778A (ND4)	—	Complex I (50% ↓)	71
LHON	Lymphoblasts	G11778A (ND4)	NA	Complex I (20% ↓) and O ₂ consumption rate (36% ↓)	72, 73
LHON	Lymphoblasts	14484C (ND6)	NA	O ₂ consumption rate (15% ↓)	73
CPEO	Muscle	4,977-bp deletion	+	Complexes I, I+III, II+III, and IV	74
CPEO	Muscle	4,977-bp deletion	+	Complex IV	75, 76
CPEO	Cybrids	4,977-bp deletion	NA	Complex I+III (3% ↓), II+III (18% ↓), and IV(48% ↓)	76, 77
KSS	Muscle	4,977-bp deletion	+	Complexes I, III and IV	78
KSS	Myoblast	4,977-bp deletion	+	Complexes I (26% ↓) and IV (56% ↓)	70
NARP	Muscle	T8993G (ATPase 6)	—	Complexes I and V	79
NARP	Lymphoblasts	T8993G (ATPase 6)	NA	Complex V (58% ↓)	80
Leigh syndrome	Muscle	T8993G (ATPase 6)	—	PDH and Complex IV	81
Leigh syndrome	Skin fibroblasts	T8993G (ATPase 6)	NA	ATP synthesis rate (80% ↓)	82

Patients with mitochondrial diseases were aged from 30 to 55 years. “↓” indicates % of decrease in enzymatic activity; “+” indicates positive results; “—” indicates negative results. Abbreviations: RRF, ragged red fibers; PDH, pyruvate dehydrogenase; NA, not available.

mtDNA mutation-induced inefficiency of mitochondrial respiration could further produce more ROS, which in turn further enhance oxidative damage to various biomolecules in mitochondria. This “vicious cycle” is propagated in typical mitochondrial diseases and results in the widely observed accumulation of oxidative damage and mutation of mtDNA, which ultimately leads to a progressive decline in the bioenergetic function of affected tissue cells in patients with mitochondrial diseases [30, 31]. We have hypothesized that increase of oxidative stress and oxidative damage are involved in the deterioration of bioenergetic function of the affected tissues in patients with mitochondrial diseases [32–34]. Recent studies conducted in other laboratories have provided compelling evidence to support the notion that oxidative stress elicited by impairment of the respiratory chain in the affected tissues of patients plays a role in the pathophysiology and progression of mitochondrial diseases [35–38].

3.3 MITOCHONDRIAL DNA MUTATION AND MITOCHONDRIAL DISEASES

The striking feature of mitochondrial disorders caused by mtDNA mutations is their clinical heterogeneity, ranging from single-organ involvement to severe multi-system disorders [39]. The same mutation of mtDNA or different mutations in the same mtDNA genes may

present with different clinical features, while the same clinical phenotype may arise from different mutations of mtDNA. In addition, the onset of clinical symptoms and phenotypic variability for mitochondrial diseases caused by the pathogenic mtDNA mutations are governed by a number of factors, including the threshold effect and mitotic segregation [11]. Most importantly, mitochondrial disorders caused by mtDNA mutations are mostly transmitted by maternal inheritance, but not all the children display symptoms, and some are even spared.

3.3.1 Heteroplasmy of mtDNA Mutations

The majority of mtDNA mutations in affected tissues are heteroplasmic, and the ratio of wild-type to mutant mtDNA may determine the onset of clinical symptoms, which is called the threshold effect of an mtDNA mutation [40]. A certain proportion of mtDNA with a pathogenic mutation is required to cause mitochondrial defects in affected tissues, and this threshold level differs with different tissues. Generally, it is suggested that the threshold level is lower in tissues that are highly dependent on aerobic metabolism than in tissues that can rely on anaerobic glycolysis for supply of ATP [41]. However, solid evidence is lacking to support a good correlation between clinical severity and the proportion of a pathogenic mtDNA mutation. On the other hand, the phenomenon of mitotic segregation can also partly explain the markedly different levels of mutated mtDNA in different

members of the same family and among different tissues of an affected individual [42]. During mitosis, heteroplasmic mutations of mtDNA may be randomly segregated in an inter- or intramitochondrial manner to each daughter cell, in which the proportion of mutant mtDNA can thus be shifted. The mitotic segregation of mtDNA mutation can explain the differential spectrum of phenotypic manifestation of the mutated mtDNA between different tissues and at different stages of life.

3.3.2 mtDNA Mutation-Related Mitochondrial Diseases

The presence of a particular heteroplasmic mutation of mtDNA strengthens the argument that the specific mutation of mtDNA is pathogenic [43]. Pathogenic mtDNA mutations include base substitutions (point mutations) and rearrangements (deletion and duplication) in the mitochondrial genome. More than 250 mutations of mtDNA have been reported to be associated with mitochondrial diseases including MERRF, MELAS, LHON, NARP, and CPEO syndromes [11, 44]. Mutations in protein-coding genes of mtDNA have been mainly documented in patients with LHON and NARP or Leigh syndrome. LHON is characterized by acute or subacute blindness that presents in early adulthood and usually affects males [45]. Over 20 point mutations of mtDNA have been associated with LHON, with most of the gene products constituting complex I. The primary mutations include the G14459A and the T14484C transitions in the *ND6* gene, the G11778A transition in the *ND4* gene, and the G3460A transition in the *ND1* gene. On the other hand, NARP is characterized by neurogenic muscle weakness, sensory neuropathy, ataxia, and retinitis pigmentosa and may associate with learning difficulty or dementia [46]. The most common mutation associated with this disease is the T8993G transversion or T8993C transition in the *ATP6* gene, which impairs the assembly of ATP synthase (complex V). It is noteworthy that the heteroplasmy of T8993C/G mutation of mtDNA would result in different clinical phenotypes depending on the mutation load [47]. The clinical features of NARP are observed with a good correlation between mutant load and clinical severity when the proportion of mutated mtDNA is lower than 75%. However, when the T8993G mutation reaches higher than 95%, it can cause Leigh syndrome, an often early-onset and lethal disorder associated with ataxia, hypotonia, spasticity, developmental delay, optic atrophy, and ophthalmoplegia [48].

The mtDNA mutations that affect overall mitochondrial protein synthesis include point mutations in tRNA genes or large-scale deletions of mtDNA, which remove one or more tRNA genes and structural genes [49]. The most common A3243G mutation in the tRNA^{Leu} gene is

associated with MELAS [50], and the A8344G mutation in the tRNA^{Lys} gene is responsible for the pathogenesis of MERRF [51]. MELAS is characterized by mitochondrial encephalopathy, repeated stroke-like episodes, seizures, and lactic acidosis, while MERRF is characterized by myoclonus, seizures, mitochondrial myopathy, cerebellar ataxia, peripheral neuropathy, and multiple lipomas. Although the clinical features and biochemical defects associated with the MELAS- and/or MERRF-specific mutations of the tRNA genes have been determined, it is still unclear as to why and how they result in such different clinical phenotypes [52]. On the other hand, diseases associated with mtDNA rearrangements mostly refer to CPEO [53] and KSS [54]. A most common defect in mtDNA is a single large-scale deletion (4,977-bp deletion), which removes one or more tRNA genes and mitochondrial protein-coding genes. Patients with CPEO and KSS present with early-onset ophthalmoplegia, ptosis, and retinitis pigmentosa, cerebellar ataxia, cardiac conduction block, renal dysfunction, and diabetes mellitus as common symptoms. Importantly, the proportion of mtDNA with a large-scale deletion has been found to increase over time in affected skeletal muscle and to accompany the progression of the disease [55].

3.4 BIOCHEMICAL CONSEQUENCES OF mtDNA MUTATION IN MITOCHONDRIAL DISEASES

Defects in the structure or function of mitochondria caused by mtDNA mutation have been shown to be associated with a wide spectrum of clinical phenotypes. There are well-documented reports of mitochondrial dysfunction in affected tissues of patients with mitochondrial diseases. In addition, mitochondrial dysfunction-elicited oxidative stress has been proven to be involved in the pathogenesis and progression of mitochondrial diseases. Furthermore, defects in glucose metabolism and the biosynthesis of heme and iron-sulfur (Fe-S) clusters, have recently been revealed in some mitochondrial diseases. Accordingly, we summarize the findings from our own and other laboratories about the relationship between the clinical phenotype and mitochondrial dysfunction in patients with mtDNA mutation-related mitochondrial diseases (Table 3.1).

3.4.1 Defects in the OXPHOS System

In a muscle pathology laboratory, defects in mitochondrial function have been determined by histological and histochemical techniques based on dye staining for specific mitochondrial enzyme activities. Alterations in the activities of cytochrome *c* oxidase (COX, complex IV) and

succinate dehydrogenase (SDH, complex II) are most often detected in affected muscles [56, 57]. Usually, the SDH activity stain clearly reveals the subsarcolemmal accumulation of mitochondria (SDH positive), and the activity assay of COX is particularly useful in the evaluation of mitochondrial myopathies (COX negative) [58]. In addition, the so-called ragged red fibers (RRF) can be found in the affected muscle fibers by Gomori trichrome stain, which shows the subsarcolemmal accumulation of abnormal mitochondria [59, 60]. On the other hand, assay of the rate of mitochondrial oxygen consumption with a Clark oxygen electrode and measurement of specific activities of the respiratory enzyme complexes spectrophotometrically have been widely applied in the diagnosis of mitochondrial diseases and in the study of the biochemical consequences of a pathogenic mutation of mtDNA [61, 62]. More recently, the Seahorse XF24 Extracellular Flux analyzer has been employed to measure metabolic profiles [oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)] and to monitor the switch of aerobic metabolism to anaerobic metabolism as a response to mitochondrial dysfunction in cells harboring a pathogenic mtDNA mutation [63]. We have suggested that the decrease of ATP generation caused by defective mitochondria can induce a compensatory upregulation of the glycolytic pathway, thus increasing the dominance of glycolysis in the cellular ATP production. It has been reported that a decrease in mitochondrial respiration and oxidative phosphorylation with a concomitant increase of glycolysis represents a hallmark of mitochondrial diseases [64, 65].

3.4.2 Oxidative Damage to Affected Tissues

A great deal of research results has suggested that mtDNA mutation-elicited ROS and oxidative damage play an important role in the pathogenesis and progression of mitochondrial diseases (Table 3.2). For example, in patients with MELAS, MERRF, LHON, or CPEO syndromes the intracellular levels of H_2O_2 and oxidative damage to DNA and lipids have been found to be increased in the primary culture of skin fibroblasts [83–86]. Majora and colleagues [87] also reported that the level of mitochondrial ROS in skin fibroblasts of KSS patients was 2.5-fold higher than that of normal control subjects. In addition, the oxidative damage to DNA in muscle of CPEO patients is much more extensive than that in muscle of patients with other types of diseases that are not associated with neuromuscular disorders [88, 89]. Moreover, Piccolo and colleagues [90] reported that lipid peroxides and fluorescent adducts of organic aldehydes with plasma proteins in blood of patients with CPEO syndrome were elevated compared with those of normal subjects. Similarly, in blood cells of patients with

MELAS-related mitochondrial disorders and patients with LHON, telomere was found to be shortened by ROS, which suggests that systematic oxidative stress is elevated in patients with mitochondrial diseases [91]. Notably, another study revealed that the skeletal muscle with a significant increase of 8-hydroxy-2'-deoxyguanosine (8-OHdG) content displayed a large amount of RRFs in patients with KSS or CPEO syndrome and that there was an excess amount of hydroxyl radicals and aldehydic lipid peroxidation products in cultured skin fibroblasts [92]. Recently, van Eijnsden et al. [93] reported that oxidative damage to proteins and the content of superoxide anions in the muscle biopsies of patients with MELAS syndrome were significantly increased compared with those of age-matched normal subjects.

To understand the biochemical consequences of a pathogenic mtDNA mutation, a promising cell model has been established through the fusion of enucleated cytoplasts (derived from patients with mtDNA mutations) with immortalized human cell lines that are devoid of endogenous mtDNA (Rho zero cells, ρ^0) [94]. Many lines of research have suggested that cybrids are an excellent tool for studying the biochemical and pathological consequences of varying proportions of a specific mtDNA mutation [95–97]. Recently, we investigated oxidative modification to mitochondrial proteins in cybrid cells harboring the A8344G mutation of mtDNA from an MERRF patient by two-dimensional gel and proteomic techniques [34]. A total of 16 carbonylated mitochondrial proteins were identified in the MERRF cybrids compared with the wild-type cybrids. It is worth mentioning that the voltage-dependent anion channel (VDAC), aconitase, and prohibitin (PHB) were quite susceptible to oxidative damage in the MERRF cybrids [34]. VDAC is a major component of the permeability transition pore complex on the outer mitochondrial membrane, which regulates the transport of ions and metabolites in and out of the mitochondria. We speculate that the accumulated oxidative damage to VDAC may cause a loss of bidirectional fluxes of ions and metabolites across mitochondrial membranes, which in turn leads to bioenergetic breakdown and elicits the pathological changes and clinical manifestations of MERRF syndrome [98]. Under normal physiological conditions, PHB acts as a chaperone to prevent misfolding of mitochondrial proteins, and thus damage to the PHB protein could inhibit its function and aggravate the mitochondrial dysfunction [99].

3.4.3 Deficiency in Iron Metabolism and Heme Synthesis

The involvement of mitochondria in the biosynthesis of heme and iron-sulfur (Fe-S) clusters underscores the

TABLE 3.2 Accumulated oxidative damage and alterations of antioxidant enzymes in affected tissues or cells from patients with mitochondrial diseases

Patients	Biopsies	Oxidative damage	Redox status and antioxidant enzymes	Reference
MERRF	Muscle	NA	Mn-SOD ↑ ^c ; GSH ↑ ^c	115
MERRF	Blood	Plasma F ₂ -isoprostane (1.6-fold ↑)	NA	136
MERRF	Cybrids	Carbonylated VDAC (2.0-fold ↑) ^b ; PHB (1.9-fold ↑) ^b	Mn-SOD ↑ ^b ; Cu,Zn-SOD (–) ^b	34
MERRF	Cybrids	H ₂ O ₂ (1.7-fold ↑)	Mn-SOD (1.7-fold ↑) _a ; CAT (1.7-fold ↑) _a Cu,Zn-SOD (1.5-fold ↑) _a	121
MERRF	Skin fibroblasts	Mitochondrial aconitase (40% ↓) _a ; heme <i>c</i> (70% ↓) _b	NA	106, 107
MELAS	Muscle	8-OHdG ↑ ^c ; TUNEL-positive ^c	Mn-SOD ↑ ^c ; GSH ↑ ^c	115, 24
MELAS	Myoblasts	8-OHdG ↑ ^c ; 4-HNE ↑ ^c	Mn-SOD (1.5-fold ↑) _a ; Catalase (2.2-fold ↑) _a	113, 104
MELAS	Brain	8-OHdG ↑ ^c	Mn-SOD and Cu,Zn-SOD (↓) ^c	84
MELAS	Cybrids	H ₂ O ₂ (1.8-fold ↑)	Mn-SOD (1.7-fold ↑) _a ; CAT (2.0-fold ↑) _a Cu,Zn-SOD (1.6-fold ↑) _a	121
CPEO	Muscle	8-OHdG of m ^a ↑DNA (40-fold ↑)	Mn-SOD ↑ ^c ; GSH ↑ ^c	115, 85
CPEO	Muscle	8-OHdG ↑ ^c ; 4-HNE ↑ ^c ; Bcl-2? ↓ ^c ; Caspase 3 ↑ ^c	Mn-SOD ↑ ^c ; Cu,Zn-SOD (–) ^c	137
CPEO	Muscle fibroblasts	8-OHdG (8.7-fold ↑); H ₂ O ₂ (1.9-fold ↑); O ₂ – (1.6-fold ↑)	Mn-SOD (2.2-fold ↑) _a ; CAT and GPx (–) _a	88
KSS	Muscle	NA	Mn-SOD ↑ ^c ; CAT ↑ ^c ; GPx ↑ ^c	138, 113
LHON	Skin fibroblasts	4-HNE (2.5-fold ↑); MDA (2.4-fold ↑)	Mn-SOD (2.9-fold ↑) _a ; Cu,Zn-SOD (–) _a	116
LHON	Blood	Leukocyte 8-OHdG (4.3-fold ↑)	NA	83
LHON	Cybrids	NA	GPx (50% ↓) _a ; GR (34% ↓) _a ; Mn-SOD (50% ↓) _a ; Cu,Zn-SOD (38% ↓) _a	118
NARP	Lymphoblasts	H ₂ O ₂ (1.5-fold ↓)	Mn-SOD (2.1-fold ↓) _a ; CAT (–) _a ; Cu,Zn-SOD (2.8-fold ↓) _a	139
NARP	Cybrids	H ₂ O ₂ (3.0-fold ↑); MDA (2.3-fold ↑); 4-HNE (2.0-fold ↑)	Mn-SOD (2.8-fold ↑) _a ; Cu,Zn-SOD (–) _a	140
Leigh syndrome	Skin fibroblasts	H ₂ O ₂ (1.6-fold ↑)	Mn-SOD (34% ↓) _b	86

Patients with mitochondrial diseases were aged between 30 and 60 years. “↑” indicates % of increase and “↓” indicates % of decrease in each of the parameters. “–” indicates no change in the indicated parameter. Superscripts a, b, and c indicate changes in the activity, protein expression, and immunohistochemistry results. Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; F₂-isoprostane, non-cyclooxygenase-derived prostanoids; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; NA, not available.

importance of these prosthetic groups in mitochondrial dysfunction of patients with mitochondrial diseases. It has been documented that deficiency in Fe-S clusters such as aconitase and subunits of complexes I and II are involved in mitochondrial encephalomyopathies and myopathies [100, 101]. In addition, a recent study demonstrated that mitochondrial dysfunction in myelodysplastic syndromes could be linked to the impairment of iron metabolism and heme biosynthesis in mitochondria [102]. Recently, heme deficiency has been considered as a vital factor in the mitochondrial decay, which is involved in the aging process and the pathogenesis of neurodegenerative diseases such as Friedreich ataxia (FRDA), AD, and PD [103]. To maintain cellular iron (Fe²⁺) homeostasis, iron regulatory proteins (IRP1 and IRP2) regulate the expression of some iron-containing proteins required for

mitochondrial function (e.g., aconitase and SDH) or cytosolic Fe²⁺ metabolism that includes transferrin receptor (TfR1) and ferritin through binding to the iron-responsive element (IRE) on the target mRNAs [104]. Mitochondrial aconitase (m-aconitase) functions in the TCA cycle to facilitate the dehydration-hydration reaction that reversibly converts citrate to isocitrate. Another isozyme is the bifunctional IRP1 that has cytosolic aconitase (c-aconitase) and IRE binding activities [105]. Therefore, the iron-dependent regulation of aconitase via IRP1 and the dependence of mitochondria on iron metabolism provide regulatory links between Fe²⁺ homeostasis and energy metabolism. It is rationalized that a disturbance in Fe²⁺ homeostasis can lead to mitochondrial dysfunction accompanied by the increase in the production of hydroxyl radicals via the Fenton reaction [24, 106].

Recently, we demonstrated [107] that the activities of both m- and c-aconitases were decreased, especially m-aconitase, in the primary culture of skin fibroblasts from MERRF patients with the A8344G mutation of mtDNA. In addition, we found that m-aconitase in the MERRF cybrids was more sensitive to H_2O_2 than that of the wild-type cybrids. It was shown that the loss of m-aconitase activity upon treatment of the mutant cybrids with H_2O_2 could be partially prevented by *N*-acetylcysteine (NAC), which is a precursor of glutathione that acts as the first-line intracellular antioxidant [108]. Moreover, we found a compensatory increase of m-aconitase expression but dramatic decrease of TfR1 expression in MERRF skin fibroblasts. The decrease of TfR1 may be a consequence of Fe^{2+} overload in response to elevated oxidative stress in mutant cells. It has been suggested that the oxidant-mediated Fe^{2+} overload can initiate and accelerate a vicious cycle that ultimately results in cellular damage and tissue degeneration [109]. Interestingly, we found that cytochrome *c* was decreased concurrently with the deficiency of heme *c*, suggesting that a functional abnormality of cytochrome *c* was induced in MERRF skin fibroblasts. The above-mentioned findings suggest that oxidative modification and deterioration of some proteins and enzymes that contain Fe-S clusters, such as aconitase, may also play an important role in the pathophysiology of mitochondrial diseases.

3.5 ALTERATION OF ANTIOXIDANT DEFENSE SYSTEM IN MITOCHONDRIAL DISEASES

In affected tissue cells with mitochondrial dysfunction-elicited oxidative stress, the induction of free radical scavengers is essential for efficient removal of ROS and some of the biological molecules with oxidative damage. Human cells have developed a ubiquitous antioxidant defense system during the evolutionary process to cope with the production of ROS by aerobic metabolism [110]. The antioxidant enzymes include SOD, CAT, GPx, and glutathione reductase (GR) together with a large number of low-molecular-weight antioxidants such as ascorbic acid, α -tocopherol, retinal, folic acid, lipoic acid, and glutathione (GSH), which can dispose of ROS and RNS to minimize their damaging effects. However, a decrease in low-molecular-weight antioxidants has been found in the serum of some patients with mitochondrial encephalomyopathies [111, 112]. In Table 3.2, we have compiled findings from previous studies on the changes of antioxidants status and the alterations in the expression or activity levels of antioxidant enzymes in mtDNA mutation-related mitochondrial diseases.

3.5.1 Differential Induction of Antioxidant Enzymes in Mitochondrial Diseases

Alteration in the expression of the antioxidant enzymes was first demonstrated by immunohistochemistry on skeletal muscle fibers from patients with mitochondrial encephalomyopathies including MELAS, CPEO, and KSS [113]. These findings indicate that the expression levels of Mn-SOD and, to a lesser extent, Cu,Zn-SOD are increased in RRFs with negative expression of cytochrome *c* oxidase [85, 114, 115]. It is noteworthy that the severity of cytochrome *c* oxidase deficiency was correlated with the increase of production of $\text{O}_2^{\cdot-}$ and induction of the expression of Mn-SOD [116]. Accordingly, it has been suggested that the dramatic induction of Mn-SOD in affected tissues and cells can be considered a biomarker for the onset of mitochondrial dysfunction in patients with a majority of mitochondrial diseases [115, 117]. On the other hand, a reduction in the protein expression and activity levels of Mn-SOD was observed in patients with LHON syndrome [118]. The disruption of Mn-SOD gene in the rat resulted in optic neuropathy, which was similar to the major symptom of LHON patients [119, 120]. On the other hand, the overproduction of ROS in cybrids harboring the A3243G or A8344G mutation of mtDNA led to an increase in the activities of antioxidant enzymes including SOD, CAT, and GPx [121]. Selenium (Se)-dependent and -independent GPx activities were also increased in response to the deficiency of respiratory enzymes in human myeloid leukemia U937 cells that had been exposed to mitochondrial stress such as chloramphenicol, an inhibitor of mitochondrial translation, or ethidium bromide, which depletes mtDNA [122].

In a previous study, we observed that the protein expression and activity levels of Mn-SOD, but not those of CuZn-SOD, CAT, and GPx, were increased in the primary culture of skin fibroblasts from CPEO patients compared with those of normal control subjects [88]. It is noteworthy that this imbalanced expression of antioxidant enzymes was much more pronounced in the fibroblasts cultured from muscle biopsies of patients with CPEO syndrome [123]. This might be due to higher oxidative stress in muscle fibroblasts, because the protein level of catalase of muscle fibroblasts was just half of that of skin fibroblasts. In addition, we also discovered that the upregulation of Mn-SOD in skin fibroblasts of patients with mitochondrial encephalomyopathies was associated with a dramatic increase in the expression of several matrix metalloproteinases (MMPs) due to increase of the intracellular level of H_2O_2 [124]. One of the most conspicuous features of the mitochondrial diseases in the affected cells was the striking derangement of the distribution and network of mitochondria,

which serve as a vehicle for efficient transmission of energy through the dynamic structural change of cytoskeleton in normal cells. Therefore, we suggest that the gross changes in morphology and network of mitochondria in the affected tissue cells from patients with mitochondrial disease is a result of activation of MMPs induced by enhanced oxidative stress [125, 126].

3.5.2 Regulation of Antioxidant Enzymes

A delicate balance exists between the expressions of both types of SOD and CAT plus GPx or thioredoxin reductase (TR) to confer cells with the ability to cope with oxidative stress. However, the imbalanced expression of antioxidant enzymes has repeatedly been observed in affected tissues and cultured cells from patients with mitochondrial diseases [127–129]. A low activity level of SOD relative to GPx or CAT could lead to the accumulation of ROS such as superoxide anions, while a high activity level of SOD relative to GPx or CAT could lead to an increased production of H_2O_2 . It was demonstrated that the generation of ROS was elevated in skeletal muscle of transgenic mice with either overexpression or knockout of Mn-SOD [130]. In addition, several research groups demonstrated that cells with overexpression of CuZn-SOD alone or Mn-SOD alone were all much more susceptible to DNA strand breaks and growth retardation and more easily killed by an extracellular burst of $O_2^{\bullet-}$ and H_2O_2 [131–134]. On the other hand, the elevated oxidative stress and oxidative damage in single transfected SOD clones can be diminished after double transfection of CAT or GPx into the cells [135]. Therefore, coordination in the expression of antioxidant enzymes in mammalian cells to efficiently cope with oxidative stress is essential for cells to survive. Recently, it was found that the expression of Mn-SOD is also upregulated by PGC-1 α , which is a transcription coactivator that controls mitochondrial biogenesis in response to increased energy demand such as exercise or cold exposure [34]. The research related to the regulation of antioxidant enzymes could provide useful information for the development of novel therapies to treat mitochondrial diseases.

3.6 REDOX THERAPY OF MITOCHONDRIAL DISEASES

Oxidative stress plays an important role in the pathophysiology of mitochondrial diseases, and thus reducing the deleterious effects of ROS by treatment with antioxidants can have therapeutic effects against a variety of ROS-mediated mitochondrial disorders. Indeed, coenzyme Q_{10} (CoQ $_{10}$) has been used as a remedy to treat

mitochondrial diseases, even at dosages as high as 2000 mg daily [141]. It has been well documented that CoQ $_{10}$ has dual roles as a component of the respiratory chain and as a potent ROS scavenger [142]. CoQ $_{10}$ can not only restore the mitochondrial OXPHOS system but also reduce the ROS-induced DNA damage and the proportion of apoptotic markers in affected biopsies of patients with mitochondrial diseases. In addition, a number of studies reported that pretreatment with CoQ $_{10}$ in human cells harboring mtDNA point mutations or large-scale deletion can reduce the increase of ROS and ROS-mediated cell apoptosis, which is induced by exogenous oxidative stress such as UV irradiation and H_2O_2 [143–145]. Clinically, the administration of CoQ $_{10}$ in patients with sporadic KSS or CPEO syndrome and patients with RRF in muscles associated with seizures, ataxia, or mental retardation has been demonstrated to be extremely beneficial [146, 147]. In addition, in a trial with AD and PD, CoQ $_{10}$ was found to restore the mitochondrial function and slow down the progression of the diseases [148]. Furthermore, in a study of lymphocytes from 12 patients with severe mitochondrial respiratory chain defects before and after 12 months of supplementation with a cocktail mixture including CoQ $_{10}$ (350 mg daily), L-carnitine, vitamin B complex, vitamin C, and vitamin K $_1$, it was found that there was a significant increase in the capacity for ATP synthesis in the lymphocytes after the treatment [149].

In addition to CoQ $_{10}$, there are ongoing developments of other antioxidants for the therapy of mitochondrial diseases. Pyruvate is one of the antioxidants that not only reduce the intracellular ROS level but also boost mitochondrial function through activation of the pyruvate dehydrogenase complex (PDHC) by inhibiting the PDH kinase (PDK). Recently, long-term administration of sodium pyruvate to patients with Leigh syndrome was found to improve effectively the exercise intolerance and restore the mitochondrial function in affected tissues [150]. On the other hand, ascorbic acid is another important antioxidant used for the treatment of patients with mitochondrial diseases because it can directly enter mitochondria in its oxidized form via glucose transporter 1 (Glut1) and thus protects mitochondria from oxidative injury [151]. Dr. Peterson reported that treatment of patients with MERRF or MELAS syndrome with ascorbic acid (1 g twice per day) could improve medical complications and the patients survived longer with less functional disability [152]. Recently, a so-called “vitamin cocktail” containing ascorbic acid and CoQ $_{10}$ has been developed and used for clinical treatment of mitochondrial diseases [153]. Furthermore, antioxidants including vitamin E, α -lipoic acid, glutathione, and NAC have also been shown to be effective in various animal models of mitochondrial

diseases and in cultured cells from some patients, but their clinical benefits need to be substantiated by well-designed clinical trials [154].

3.6.1 Strategy for Targeting Antioxidants to Mitochondria

The specific delivery of drugs or antioxidants into mitochondria has been proven to be a potential therapeutic approach. Recently, Murphy and Smith [155] developed one specific approach to selectively target antioxidants to mitochondria by conjugating them to lipophilic cations such as triphenylphosphonium (TPP). Lipophilic cations can pass easily through lipid bilayers because they carry a positive charge that is colocalized over a large surface area, and the electrochemical gradient drives their accumulation in mitochondria because of the large membrane potential across the mitochondrial inner membrane (150–180 mV). Therefore, the newly developed antioxidant MitoQ₁₀, a CoQ₁₀ molecule attached to the TPP⁺ ion, has been used in a wide range of mitochondrial disease models, and the results showed protection against oxidative damage [156, 157]. In fact, MitoQ₁₀ is more potent and effective than CoQ₁₀ in preventing oxidative stress-induced apoptosis in skin fibroblasts from patients with mitochondrial diseases [158]. In addition, in vivo studies revealed that MitoQ₁₀ administered to mice and rats by intravenous injection was rapidly cleared from the plasma and accumulated in the heart, brain, liver, kidney, and skeletal muscle [159] and can selectively protect mitochondria from ischemia-reperfusion injury of the heart [160]. MitoQ₁₀ is now under clinical development (in phase II human clinical trials), and orally administered MitoQ₁₀ can be applied to a wide spectrum of human pathologies that involve mitochondrial oxidative damage such as mtDNA mutation-elicited mitochondrial diseases. Similarly, the Szeto–Schiller (SS) tetrapeptides have also recently been developed as small cell-permeant antioxidants in a mitochondrial membrane potential-independent manner [161]. The structure motif of SS peptides centers on alternating aromatic (phenylalanine, tyrosine, dimethyltyrosine) and basic (arginine, lysine) amino acid residues, in which tyrosine and dimethyltyrosine residues likely act as free radical scavengers. The SS peptides have been demonstrated to reduce ROS in mitochondria and clearly showed its potential in the treatment of mitochondrial disorders or oxidative stress-related diseases such as myocardial infarction and ischemic brain injury [162, 163].

3.6.2 Antioxidant Enzymes Targeted to Mitochondria

In light of the observation of the imbalanced expression of antioxidant enzymes found in most affected tissues of

patients with mitochondrial diseases, it has been thought that increasing the expression of SOD, CAT, or GPx in affected individuals is another strategy for the therapy of mitochondrial diseases. For example, LHON cybrids with complex I deficiency were associated with decreased expression of Mn-SOD, and thus, by infection of the cells with recombinant adeno-associated virus (rAAV) containing the human Mn-SOD gene, the survival of LHON cells was increased up to 90% [108, 109]. On the other hand, the administration of SOD-CAT mimetics including manganese(III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) and EUK-418 (developed by Eukarion Inc., USA) to boost the activities of Mn-SOD and CAT could protect cells from oxidative injuries in animal models of neurodegenerative diseases including PD, AD, and ALS [164]. In addition, supplementation with Se to activate GPx and TR in cybrids harboring 98% T8993G mutated mtDNA could decrease oxidative stress and oxidative damage [165]. Recently, with the redox approach of overexpression of CAT targeted to the mitochondria (mCAT) in mitochondrial mutator mice with DNA polymerase gamma (POLG) deficiency, mice were better protected from ROS-induced mitochondrial damage and had an extension of maximum life span [166, 167]. Other studies also revealed that overexpression of antioxidant enzymes such as peroxiredoxin [168], thioredoxin [169], and GPx [170] targeted to mitochondria could effectively protect cells from exogenous stress-induced oxidative damage and cell death. Although there are few reports on the therapeutic manipulation of antioxidant enzyme in animal models, the reduction of mitochondrial ROS by overexpression of antioxidant enzymes targeted to mitochondria can be considered a novel strategy to develop effective therapies for treatment of mitochondrial diseases in the future.

3.7 GENETICS-BASED GENE THERAPY FOR MITOCHONDRIAL DISEASES

Currently, there is no cure for mitochondrial diseases, and only treatments to relieve symptoms such as antioxidant therapy can be offered. This has led researchers to consider mitochondrial gene therapy, which can be carried out either by the expression of an engineered gene product to rescue defects caused by an nDNA mutation or by import of normal copies or relevant sections of mtDNA into mitochondria [171, 172]. Although gene therapy in patients with mtDNA mutations is a challenge because of the heteroplasmy of mtDNA, this idea has been tested and proved successful in yeast from Nagley's group in 1988 [173]. They demonstrated that yeast carrying an mtDNA mutation at *MTATP8* gene could be rescued when normal copies

of the gene were introduced into the nucleus, resulting in cytosolic translation of the gene product, the wild-type protein of ATPase 8. Recently, a successful targeting of *ATPase 6* gene in a cell model was shown to partially rescue a respiratory chain-deficient phenotype caused by a pathogenic mutation in the *MTATP6* gene [174]. However, it was pointed out that the assembly of complex V was not efficient to integrate correctly with the imported protein to make a mature and functional ATP synthase. The question is how to efficiently translocate engineered polypeptides to mitochondria, although the engineered polypeptides could be targeted with the mitochondria-targeting sequence in their N-terminals [175]. Recently, Corral-Debrinski and colleagues [176] attempted to optimize the expression of highly hydrophobic mitochondrial proteins by targeting the known protein (such as Mn-SOD) that is localized to the mitochondria. The results showed that facilitated cotranslational translocation of the gene product could improve mitochondrial import of the allotopically expressed proteins, preventing the formation and accumulation of cytosolic aggregates of the overexpressed proteins.

On the other hand, import of normal tRNA is another potential therapy for treatment of patients with mtDNA mutation in a tRNA gene [177]. Bhattacharyya and colleagues [178] first isolated a large tRNA import complex (RIC) from the inner mitochondrial membrane of *Leishmania tropica* and reconstituted it into phospholipid vesicles. With the application of RIC, Mahata and co-workers [179] suggested that the RIC can enter human cells through a caveolin-1-dependent pathway, and thereby induce import of endogenous cytosolic tRNAs, including tRNA^{Lys}, and restore mitochondrial function in the cybrids harboring a mutant tRNA^{Lys} gene of mtDNA. The use of tRNA genes to rescue mitochondrial function may be beneficial for the management of such genetic disorders. Mitochondrial gene therapy has been established and tested in both cultured cells and animal models, and the experimental results have suggested that it holds some promise in clinical application for treatment of patients with mtDNA mutations in the future.

3.8 CONCLUSION

Mitochondrial diseases are caused by mutations in mtDNA and/or nDNA, but the molecular mechanisms underlying the pathogenesis of mitochondrial diseases are still poorly understood. It still remains a mystery as to how and why mutations in different genes lead to similar clinical symptoms and the same mtDNA mutations can manifest different clinical features. However,

in light of the experimental data from our own and other laboratories, we believe that bioenergetic dysfunction and accumulation of deleterious metabolic intermediates in affected tissues or cells may be involved in the onset and progression of this prominent group of metabolic diseases (Table 3.1). In addition, defective mitochondria also generate more ROS and free radicals via electron leak from the respiratory chain. As a consequence, significant increases in oxidative stress and oxidative damage are often observed in the affected tissues and peripheral blood cells of most patients with mitochondrial diseases (Table 3.2). Therefore, it has been generally established that oxidative stress and oxidative damage are involved in the pathophysiology of mitochondrial diseases [12, 180]. Once the damage persists too long or is too serious to be repaired, the mitochondria would sense and integrate the extramitochondrial stress and signal to drive the cell into an irreversible death process (apoptotic mechanism) [34, 181]. This scenario may explain the clinically well-documented age-dependent progression and worsening of disease in the majority of mitochondrial diseases. On the other hand, with the development of cDNA microarray and proteomic techniques, altered expression of several clusters of genes and protein modifications have been observed in affected tissues and cultured cells of patients. Compensatory upregulation of OXPHOS genes and oxidative stress-responsive genes has been widely investigated in affected individuals with mitochondrial dysfunction [182, 183]. Specifically, the dramatic induction of Mn-SOD observed in affected tissues can be considered as an early sign of OXPHOS deficiency in the majority of mitochondrial diseases, and such manifestations are often correlated with the mtDNA mutation load and the proportion of abnormal mitochondria [115, 117].

To counteract the effects of oxidative stress, antioxidants have been utilized in the treatment of some mitochondrial diseases. Clinical trials of CoQ₁₀ with other antioxidants such as vitamins C, E, and K as a form of “antioxidant cocktail” have been conducted to treat patients with mitochondrial diseases and neurodegenerative diseases [172]. In addition, reduction of mitochondrial ROS has been shown to be beneficial to patients with specific mitochondrial disorders. Thus the development of antioxidants targeting mitochondria such as MitoQ₁₀ has been a productive endeavor. Notably, there has been much research and development in the design of Mito-vitamin E, Mito-TEMPOL, and Mito-NAC for the treatment of diseases caused by mitochondrial disorders [184]. Therefore, targeting mitochondria with organelle-specific agents is proven to be an effective therapeutic strategy to specifically reduce the mitochondrial ROS in tissue cells of patients with a mitochondrial defect [185]. Although there have been

some designs of gene therapy to correct mitochondrial defects caused by a pathogenic mtDNA mutation, their clinical applications face many challenges. With consideration of the hurdles and potential risk of mitochondrial gene therapy, including the choice of appropriate viral or nonviral vectors, the high efficiency of the delivery of antioxidants to the affected tissues, and the low adverse (e.g., immunological) responses, redox therapy by antioxidants is a promising treatment for mitochondrial diseases and other diseases caused by mitochondrial dysfunction.

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OXIDATIVE STRESS IN KAINIC ACID NEUROTOXICITY: IMPLICATIONS FOR THE PATHOGENESIS OF NEUROTRAUMATIC AND NEURODEGENERATIVE DISEASES

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4.1 INTRODUCTION

It is well established that neural membranes are composed of glycerophospholipids, sphingolipids, cholesterol, and proteins. The distribution of lipids in two leaflets of lipid bilayer is asymmetric [1, 2]. Glycerophospholipids and sphingolipids contribute to the lipid asymmetry, whereas cholesterol and sphingolipids form lipid microdomains or lipid rafts, which float within the membrane and act as molecular sorting machines and platforms for signal transduction pathways [3, 4]. Thus lipid raft are a unique compartment of the plasma membrane, which not only ensure correct intracellular trafficking of proteins and lipids through protein-protein interactions and concentrate certain proteins in microdomains, while excluding others, but also modulate signal transduction processes associated with neural cell functions. The maintenance of transbilayer lipid asymmetry is a dynamic process, which is necessary for the maintenance of normal neural membrane function. The disruption of asymmetry through receptor activation results in neural cell activation necessary for normal cell function, but overactivation of neural membrane receptors either by agonist or through neural trauma causes neurodegeneration [5, 6].

Kainate (KA) administration in rodents has been used as an animal model to study molecular mechanism

of neurodegeneration [7]. KA administration leads to different patterns of neuronal excitation. Thus systemic injection of KA to rats produces selective neuronal vulnerability in the hippocampal hilus, CA1, and CA3 subfields, whereas granule cells in dentate gyrus are resistant to KA-induced neurotoxicity. Systemic administration of KA in adult rats also produces persistent seizures syndrome [8] and triggers acute and delayed neuronal death in the hippocampal CA1 and CA3 regions [7].

KA-mediated neurotoxicity cell death is caused by interactions between KA and KA receptors (KA-R), which are composed of five different subunits (GluR5, GluR6, GluR7, KA1, and KA2) [8, 9]. KA-Rs are classified into low-affinity receptor families (GluK1–GluK3) and high-affinity receptor families (GluK4–GluK5) based on their affinity for the neurotoxin KA. These two families share a 42% sequence identity for the intact receptor but only a 27% sequence identity at the level of extracellular amino terminal domain. These receptors allow the influx of Na^+ and the efflux of K^+ . KA-R-mediated influx of Na^+ is accompanied by the passive movement of Cl^- and water molecules into neural cells, resulting not only in the osmotic overloading of water but also in KA-R-mediated membrane depolarization producing increase in intracellular Ca^{2+} [10–12]. Nuclear microscopic high-resolution elemental maps of KA-mediated damaged area show very

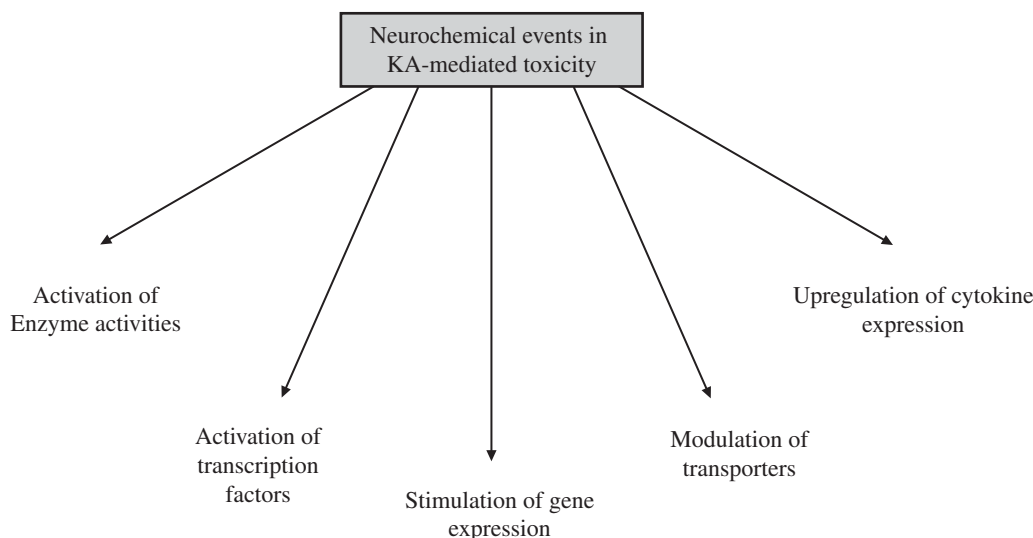


Fig. 4.1 Modulation of neurochemical activities in KA-induced neurotoxicity.

high intracellular calcium concentrations in almost all glial cells, supporting the view that KA-mediated neurotoxicity may be initiated by Ca^{2+} influx through depolarization, maintained by failure of Ca^{2+} extrusion because of ATP depletion, and intensified by increase in intracellular Ca^{2+} leading to mitochondrial dysfunction resulting in further depletion of ATP [7, 8, 13, 14]. These processes also result in the loss of Mg^{2+} blockade of the NMDA receptor, which allows further Ca^{2+} influx through NMDA receptor channel. Thus elevation in intracellular Ca^{2+} is a major component of neural cell death in KA-mediated neurotoxicity [14]. Stimulation of KA receptors results in enrichment of glycerophospholipid, sphingolipid, and cholesterol metabolism through the activation of enzyme activities, modulation of transcription factors, transporters, and gene expression resulting in increase in oxidative stress, inflammation, and neurodegeneration (Fig. 4.1) [7]. At the cellular level KA-induced neurotoxicity results in activation of microglia and astrocytes, which strongly express $\text{TNF-}\alpha$ mRNA and protein [15]. Collective evidence suggests that $\text{TNF-}\alpha$ derived from KA-activated microglia increases excitotoxic insult to hippocampal neurons, and may be responsible for the induction of neuronal apoptosis *in vitro* and *in vivo*.

4.2 GLYCEROPHOSPHOLIPID METABOLISM ALTERATIONS IN KA-INDUCED NEUROTOXICITY

From neural membrane, arachidonic acid (ARA) is released from glycerophospholipids either through the stimulation of phospholipase A_2 (PLA_2) or through the

involvement of the phospholipase C (PLC)/diacylglycerol (DAG) lipase pathway [16–18]. Stimulation of PLA_2 and PLC/DAG lipase pathway results in the generation of high levels of eicosanoids (prostaglandins, leukotrienes, and thromboxanes) and reactive oxygen species (ROS). ROS include oxygen radicals, superoxide anions, hydroxyl, alkoxyl, and peroxy radicals, and hydrogen peroxide. Systemic administration of KA into adult rats increases c PLA_2 activity in rat brain homogenate and immunoreactivity in neurons at 1 and 3 days after injection [19] (Fig. 4.2). KA injections increase the c PLA_2 immunoreactivity in astrocytes after 1, 2, 4, and 11 weeks. Increased c PLA_2 activity in neurons in KA-mediated toxicity may be involved in neurodegeneration, whereas the elevation of c PLA_2 immunoreactivity in astrocytes is associated with gliosis [19]. Injections of KA also induce a marked increase in c PLA_2 mRNA and protein levels [17, 19, 20], and c PLA_2 inhibitors [20] block the increase in PLA_2 activity by inhibiting expression of c PLA_2 mRNA. These observations suggest that generation of ARA is a receptor-mediated process. In addition, COX-2 immunoreactivity is also pronouncedly enhanced, particularly in CA3 pyramidal not only in neurons and activated microglial cells but also in activated astrocytes along with the increase in expression of the microglia-specific protein Iba1 and increase in synthesis of prostaglandin E_2 (PGE_2) [19, 21]. Collective evidence suggests that elevated levels of ARA and its metabolites (eicosanoids) produce a variety of detrimental effects on neural membrane structures, activities of membrane enzymes, generation of ROS, and neurotransmitter uptake systems [7, 14].

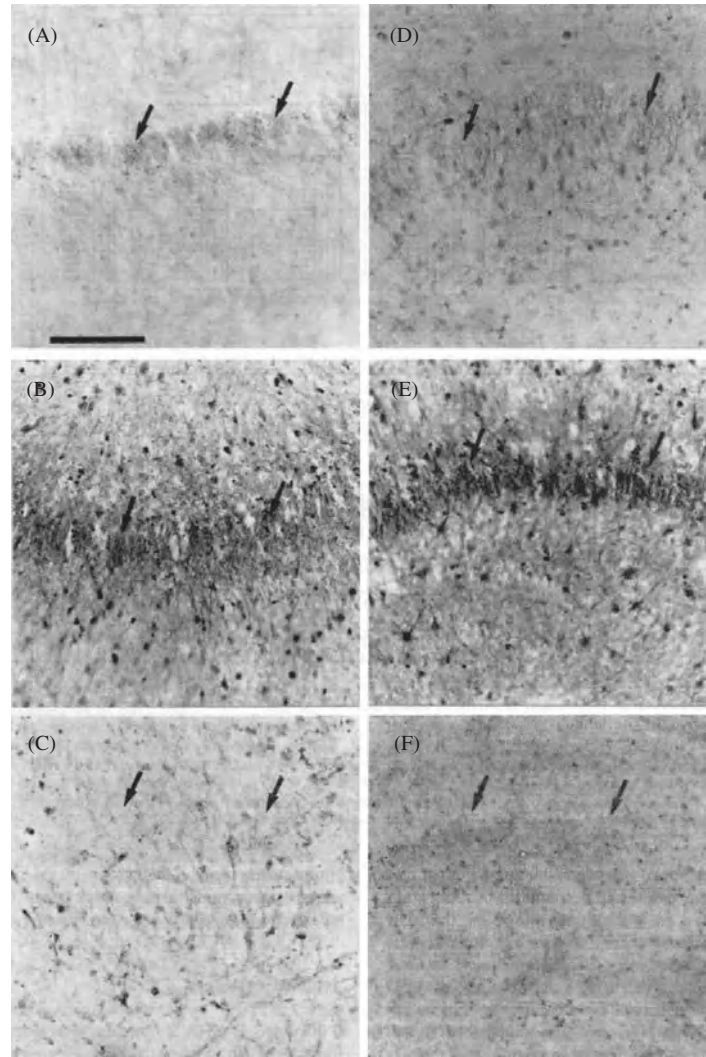


Fig. 4.2 Effect of KA-induced neurotoxicity on cPLA₂ and 4-HNE immunoreactivity in CA1 hippocampal subfield. Slices of CA1 subfield of the rat hippocampus are immunostained with monoclonal antibody to cPLA₂ (A, B, and C) and 4-HNE monoclonal antibody (D, E, and F). A and D are untreated control slices. B and E are slices that have been stained with 1 mM KA, followed by fixation and immunocytochemical staining 7 days after treatment. C and F are slices that have been treated with 1 mM KA, followed by addition of quinacrine 3 h later and fixation and immunocytochemical staining 7 days later. Arrows indicate hippocampal pyramidal neurons. (A–C) Very little or no staining for cPLA₂ is observed in pyramidal neurons in control hippocampal slices (A), while an increase in staining is observed in neurons after KA treatment (B). The increase in cPLA₂ immunoreactivity is prevented by treating the slices with quinacrine after the KA application (C). (D–F) Very little or no staining for 4-HNE is observed in pyramidal neurons in the normal hippocampus (D), while an increase in staining is observed in neurons after KA treatment (E). The increase in 4-HNE immunoreactivity is prevented by treating the slices with quinacrine after KA application (F). Scale bar, 160 μ m. Reproduced with kind permission from Elsevier [Farooqui et al. *Brain Res Rev* (2001) 38: 61–78].

In KA-mediated neurotoxicity, nonenzymic peroxidation of ARA results in generation of high levels of 4-hydroxynonenal (4-HNE), resulting in increased immunoreactivity in cell bodies of neurons and the neuropil (Fig. 4.2). 4-HNE is a nine-carbon unsaturated aldehyde that not only reacts with lysine, cysteine, and histidine residues in proteins but also binds to free amino acids and deoxyguanosine [7, 22]. 4-HNE causes a number of deleterious effects in cells including inhibition

of DNA synthesis, disturbance in calcium homeostasis, and inhibition of mitochondrial respiration [7, 14]. ROS-mediated injury results from the reaction of free radical species with proteins and unsaturated lipids in plasma membrane, leading to chemical cross-linking. This depletion of unsaturated lipids is associated with an alteration in membrane fluidity, which may be responsible for changes in activity of membrane-bound enzymes, ion channels, and receptors [14]. In addition,

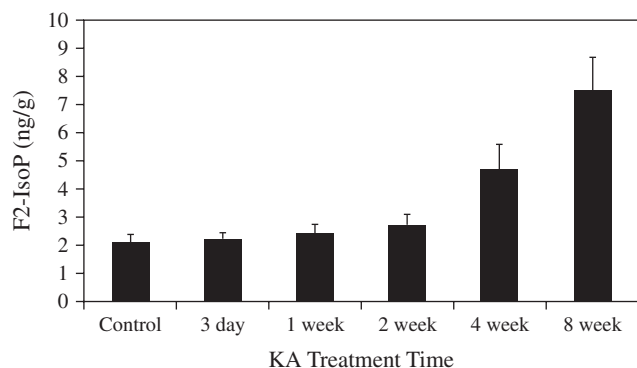


Fig. 4.3 Mean F₂-isoprostane levels in rat hippocampus under control conditions and 3 days, 1 week, 2 weeks, 4 weeks, and 8 weeks after kainate injection. $P < 0.05$ was considered significant. Four rats were used at each time point. Modified from Farooqui et al (2007a). *Brain Res Rev* 56: 443–471.

the C3 position of 4-HNE is a highly reactive site that undergoes a Michael addition reaction with cellular thiols and hence readily forms adducts with glutathione or proteins containing thiol groups. 4-HNE may produce a number of deleterious effects in cells including inhibition of DNA and RNA synthesis, disturbance in calcium homeostasis, and inhibition of mitochondrial respiration. In vivo, nonenzymic peroxidation of ARA also produces isoprostanes, which are prostaglandin-like metabolites that induce their action through isoprostane receptors, which are identical or analogous to thromboxane A₂ receptors [23]. Through a receptor-mediated process, isoprostanes modulate vasoconstriction of retinal vessel and the brain microcirculation [24]. In addition, isoprostanes can be transformed into H₂-isoprostane endoperoxides, which can give rise to cyclopentenone isoprostanes, which are very reactive α,β -unsaturated aldehydes [24]. KA injections produce no changes in F₂-isoPs levels at 3 days, 1 week, and 2 weeks after KA administration; there is a significant increase ($\sim 134\%$) in F₂-isoP levels at 4 weeks after kainic acid injection compared to controls. At 8 weeks after injection, F₂-isoP levels are increased ($\sim 180\%$) compared to those in the 4 weeks post-KA injected rats (Fig. 4.3) [25].

Treatment of hippocampal slices with KA also results in a time-dependent increase in nuclear NF- κ B levels in CA3 and CA1 areas of hippocampus, but not dentate gyrus, compared with saline-injected controls. ROS, which are generated through KA-induced neurotoxicity, also stimulate NF- κ B, a transcription factor that is present in the cytoplasm in a repressed form attached to its inhibitory protein, I- κ B (Fig. 4.4) [7]. ROS-mediated stimulation of NF- κ B-I- κ B complex facilitates

the release of NF- κ B, which is translocated to the nucleus, where it mediates the transcription of more than 150 genes including genes that code for many proinflammatory enzymes, such as sPLA₂, COX-2, iNOS, SOD (Fig. 4.5), and matrix metalloproteinases, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, cytokines (TNF- α , IL- β , IL-6, c-fos, and protooncogene Bcl-2 [7]. These parameters not only promote the onset of oxidative stress and inflammation but also modulate immune function responses that are closely associated with apoptosis [5]. ROS also promote apoptotic cell death through changes in mitochondrial permeability and poly (ADP-ribose)polymerase activation, which is accompanied by the depletion of NAD. Depletion of NAD causes reduction in ATP, leading to cell death (Fig. 4.4).

Under normal conditions, nitric oxide synthase (NOS) converts arginine into citrulline with the production of nitric oxide (NO). Citrulline is then recycled to arginine by successive actions of argininosuccinate synthetase and argininosuccinate lyase, forming the citrulline-NO cycle. Excessive stimulation of KA-R may also damage neurons through the reaction between NO and superoxide anion and the generation of peroxynitrite (ONOO⁻). ONOO⁻ not only interacts with sulfhydryl groups but can hydroxylate the aromatic rings of amino acid residues [7]. In addition, ONOO⁻ reduces mitochondrial respiration, inhibits membrane pumps, depletes cellular glutathione, and damages DNA, through the activation of poly (ADP-ribose) synthase, an enzyme that leads to cellular energy depletion [26]. Furthermore, ONOO⁻ not only interferes with key enzymes of the tricarboxylic acid cycle, the mitochondrial respiratory chain, and mitochondrial Ca²⁺ metabolism, but also reacts with lipid, proteins, and DNA [27]. All these processes contribute to neuronal energy deficiency and neurotoxicity caused by KA.

4.3 SPHINGOLIPID METABOLISM ALTERATIONS IN KA-INDUCED NEUROTOXICITY

Intracerebroventricular injections of KA in rats produce a significant increase in ceramide immunoreactivity and levels in the hippocampus at 1 day and 3 days after injection compared to controls. This increase in ceramide may be due to either enhancement in the de novo synthesis of ceramide or increase in hydrolysis of sphingomyelin by sphingomyelinase. Tandem mass spectrometric profiling of lipid extract from KA-injected and control hippocampal tissues indicates significant increase in ceramide with different molecular species including 16:0, 18:0, 20:0, 22:0, and 24:1 fatty acids in KA-injected hippocampus

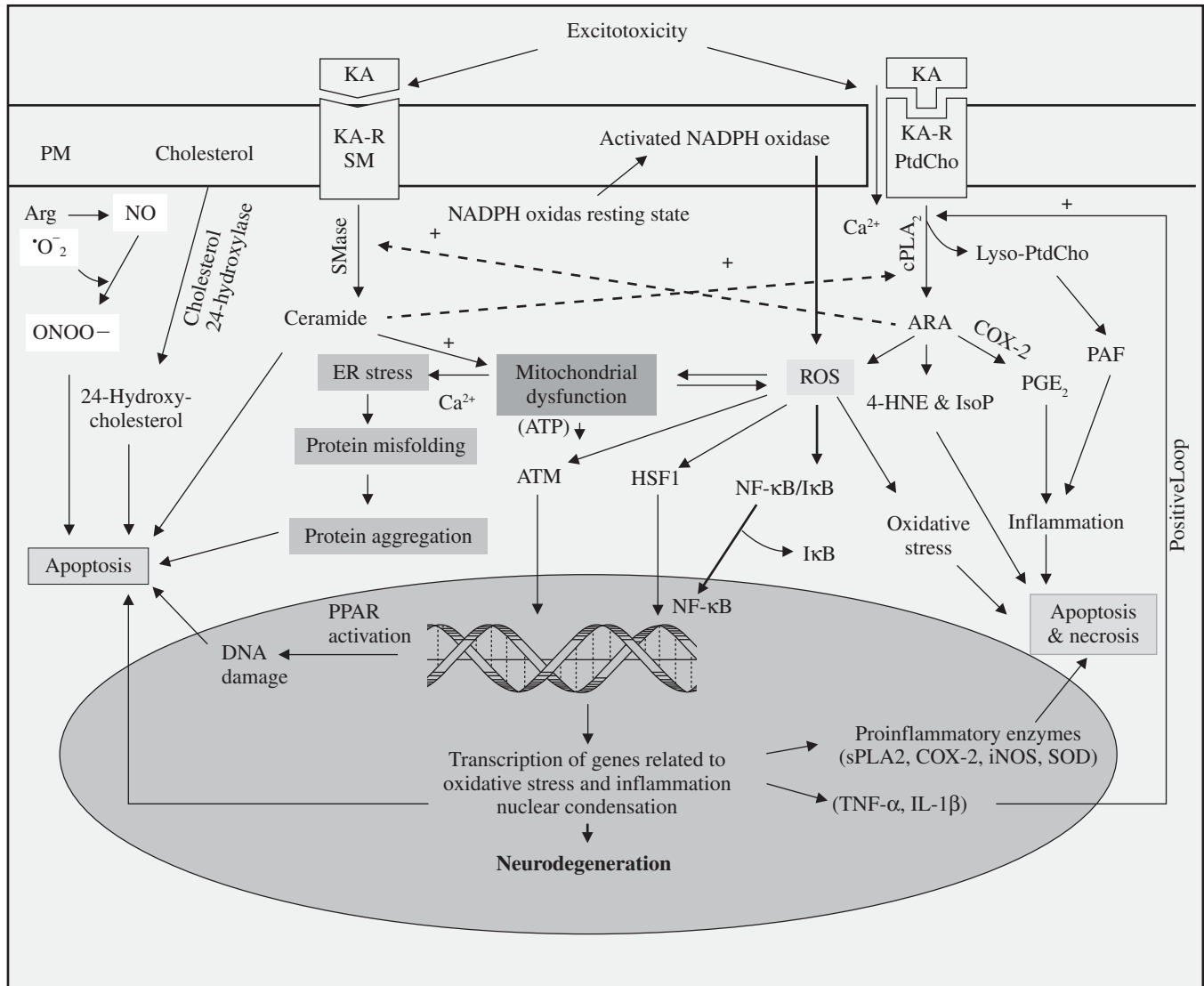


Fig. 4.4 Diagram showing interactions between glycerophospholipid and sphingolipid lipid-derived lipid mediators. KA, kainate; KA-R, kainate receptor; SM, sphingomyelin; SMase, sphingomyelinase; cPLA₂, cytosolic phospholipase A₂; PtdCho, phosphatidylcholine; ARA, arachidonic acid; Lyso-PtdCho, lyso-phosphatidylcholine; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; SOD, superoxide dismutase; 4-HNE, 4-hydroxynonenal; PGE₂, prostaglandin E₂; PAF, platelet-activating factor; IsoP, isoprostane; Arg, L-arginine; NO, nitric oxide; ONOO⁻, peroxynitrite; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; PARP, poly(ADP-ribose) polymerase; ATM, Ataxia-telangiectasia mutated; HSF-1, heat shock transcription factor 1. Positive sign (+) indicates stimulation. (See color insert.)

[28]. The increase in ceramide levels is associated with increased expression and activity of serine palmitoyl-transferase (SPT) after KA injections. Immunohistochemical analyses indicate baseline expression of SPT in neurons and gradual increase in immunoreactivity in astrocytes after KA treatment [29]. The expression of SPT in reactive astrocytes suggests that these cells may be involved in turnover of sphingolipids and generation of ceramide in KA-induced excitotoxic brain injury. In addition, neurons lacking acid sphingomyelinase exhibit decrease in vulnerability to excitotoxicity, which is

associated with decreased levels of intracellular calcium and oxyradicals [30]. Accumulating evidence suggests that in KA-mediated neurotoxicity, increase in ceramide may facilitate the opening of the mitochondrial permeability transition pores, which disrupts the transmembrane potential, causing the release of cytochrome *c*, caspase-3 activation, and activation of PLA₂ resulting in apoptotic cell death [7]. Inhibition of SPT by L-cycloserine or myriocin produces a significant neuroprotective effect for a short time after KA-induced toxicity [29]. In addition, studies on temporal changes in sphingosine

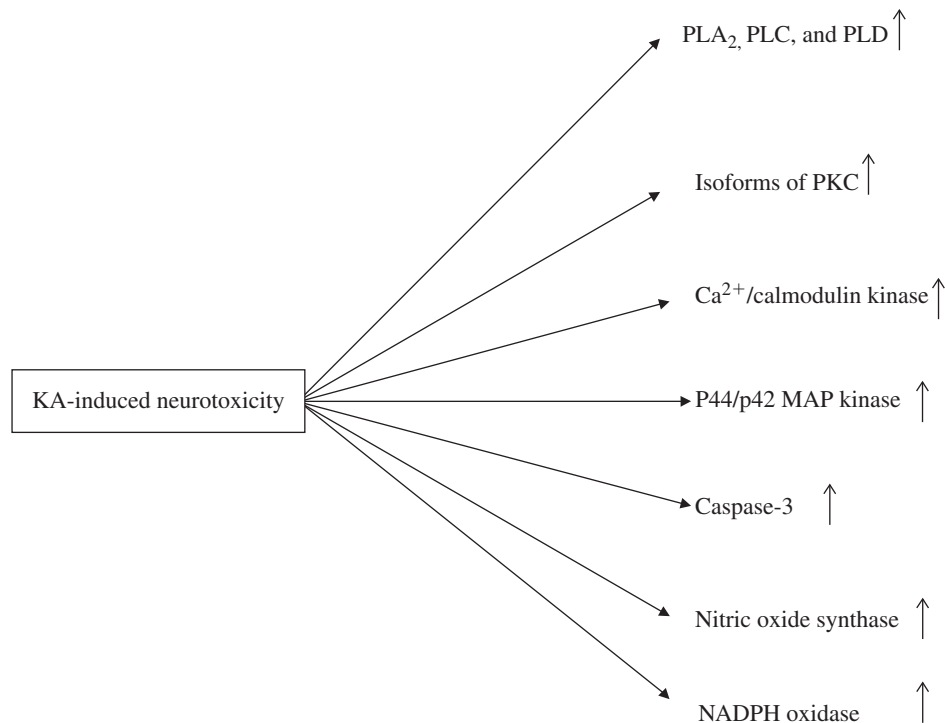


Fig. 4.5 Increase in enzyme activities following KA-induced neurotoxicity.

kinase 1 (SPHK1)/sphingosine 1 phosphate receptor 1 (S1P₁) in mouse hippocampus during KA-induced neurotoxicity indicate that the lowest level of SPHK1 protein expression is found 2 h after KA treatment. Six hours after KA treatment, the expression of SPHK1 and S1P₁ proteins steadily increases in the hippocampus. Immunohistochemical analysis indicates that SPHK1 and S1P₁ are more immunoreactive in astrocytes within the hippocampus of KA-treated mice than in hippocampus of control mice. These results indicate that the SPHK1/S1P₁ signaling axis may play an important role in astrocyte proliferation during KA-induced excitotoxicity [31].

4.4 CHOLESTEROL METABOLISM ALTERATIONS IN KA-INDUCED NEUROTOXICITY

As stated above, cholesterol is an integral component of neural membranes. It is crucial for the function of neuronal and glial cells in brain [32, 33]. Cholesterol not only modulates the physicochemical properties of neural membranes but also regulates endocytosis, antigen expression, and activities of membrane-bound enzymes, receptors, and ion channels [32, 33]. Although neurons synthesize sufficient quantities of cholesterol to survive and grow, at the time of synaptogenesis cholesterol is synthesized by glial cells and delivered to neurons by

cholesterol-transporting proteins, such as apoE and ATP binding cassette proteins (ABCA1 and ABCG1) [33].

In brain, cholesterol is metabolized to oxysterols through hydroxylation reactions, and the occurrence of 24-, 27-, and 7-hydroxycholesterol has been well established in brain tissue and CSF samples [34]. Intracerebroventricular injections of KA in rats results in an increase in immunoreactivity to cholesterol in the affected CA fields of the hippocampus. The increase is confirmed by increased filipin staining of cholesterol in adjacent sections from the same animals and in hippocampal slice or neuronal cultures after KA treatment [20]. The increase in cholesterol staining in slice cultures suggests that increase in cholesterol in neurons after KA treatment is not the result of exogenous transfer from the bloodstream. Likewise, increase in cholesterol staining in individual neurons indicates that increase in neuronal cholesterol staining is likely due to either an increase in cholesterol biosynthesis or a defect in cholesterol export in neurons themselves. In neuronal cultures, addition of lovastatin, an inhibitor of cholesterol synthesis, attenuates the increased filipin staining after KA treatment. The increase in brain cholesterol therefore points to a local disturbance in cholesterol homeostasis [20]. This increase in cholesterol level in KA-treated brain is accompanied by an increase in cholesterol oxidation products, such as 7-ketocholesterol and 24-hydroxycholesterol. These metabolites not only produce neurotoxic effects but also induce exocytosis when applied externally to PC12 cells, as

determined by capacitance measurements under patch-clamp conditions and total internal reflection fluorescence microscopy (TIRFM) of labeled vesicles [35]. The effect of 7-ketocholesterol is dependent on the integrity of lipid rafts. Enhanced exocytosis induced by the oxysterol can be abolished by pretreatment of cells with methyl cyclodextrin, which chelates cholesterol and disrupts rafts. Similar effects on enhanced exocytosis are observed after external application of several ceramide species, including C18:0 ceramide, and, as with oxysterols, the effect is raft dependent. These results indicate that increased oxysterol [36] and ceramide [28, 29] levels in the hippocampus following KA-mediated neurotoxicity may enhance neurotransmitter release through exocytosis and further propagate excitotoxic brain injury [29, 36, 38]. Furthermore, 7-ketocholesterol contains a reactive keto group and may form adducts with the amino group of other lipid and protein components of neural membranes. Studies on metabolism of 27-, 25-, and 24-hydroxycholesterol in cultures of rat astrocytes, Schwann cells, and neurons indicate that 27- and 25-hydroxycholesterol, but not 24-hydroxycholesterol, undergo a 7 α -hydroxylation with subsequent oxidation to 7 α -hydroxy-3-oxo- δ 4 steroids in all three cell types [37]. All these oxysterols behave as neurotoxic agents toward the human neuroblastoma cell line SH-SY5Y and induce apoptosis, as indicated by DNA-fragmentation, caspase-3 activation, and a decrease of the mitochondrial membrane potential.

The generation of high levels of oxysterols (hydroxycholesterols) in KA-mediated neurotoxicity modulates neural cell survival. Oxysterols also exert tight control over neural cell cholesterol trafficking by altering cholesterol influx/efflux [38]. Oxysterols not only regulate Ca^{2+} signals, modulate cPLA₂ activity, and block the phosphorylation of endothelial NOS but also interact with lipid metabolites of glycerophospholipid and sphingolipid metabolism [39–41]. In addition, some oxysterols trigger the stimulation of NADPH oxidase, generation of superoxide anions, loss of mitochondrial

transmembrane potential ($\Delta\Psi_m$), release of cytochrome *c*, and activation of caspase-3. These processes are closely associated with apoptotic cell death [42]. Furthermore, some hydroxycholesterols, such as 7 β -hydroxycholesterol not only retard the secretion of soluble amyloid precursor protein (APP) from cultured rat hippocampal H19-7/IGF-IR neuronal cells but also inhibit α -secretase activity [43]. 7 β -Hydroxycholesterol also inhibits protein kinase C- α , an enzyme critical in memory consolidation and synaptic plasticity. Low levels of 7-hydroxycholesterol modulate normal APP processing, but high levels of hydroxycholesterol promote the formation of A β that may contribute to the oxidative stress and neural cell loss observed in Alzheimer disease (AD) [43]. Levels of cholesterol and oxysterol in KA-induced neurodegeneration in the hippocampus are affected by intraperitoneal injections of a blood-brain barrier-permeant statin, lovastatin. Modulation of excessive increase in cholesterol and oxysterol levels in brain tissue after statin treatment correlates with increased survival of hippocampal pyramidal neurons, suggesting that reduction in oxysterols may be an important mechanism for the neuroprotective effect of statins [28, 29, 44].

4.5 CONSEQUENCES OF INTERACTIONS AMONG GLYCEROPHOSPHOLIPID-, SPHINGOLIPID-, AND CHOLESTEROL-DERIVED LIPID MEDIATORS IN KA-MEDIATED NEUROTOXICITY

As stated above, biophysical properties of neural membranes are maintained by a specific physiological glycerophospholipid, sphingolipid, and cholesterol composition [41]. KA-mediated neurotoxicity results not only in the enrichment of glycerophospholipid, sphingolipid, and cholesterol metabolism but also in generation of high levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators (Table 4.1) [7]. As

TABLE 4.1 Alterations in levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in KA-induced neurotoxicity and ischemia

Lipid Mediator	KA-Mediated Toxicity	Neurotraumatic Diseases	Neurodegenerative Diseases	Reference
Fatty acids (arachidonic acid)	Increased	Increased	Increased	14, 46
Prostaglandins	Increased	Increased	Increased	14, 46
4-HNE	Increased	Increased	Increased	6, 14, 60
Isoprostanes	Increased	Increased	Increased	61, 62
Ceramide	Increased	Increased	Increased	28, 63–67
7- β -Hydroxy-cholesterol	Increased	—	Increased	20, 43, 68
7-Keto-cholesterol	Increased	—	Increased	44, 69, 70
24-Hydroxy-cholesterol	Increased	No effect	Increased	44, 69, 70
22(<i>R</i>)-hydroxycholesterol	—	—	Decreased	71

mentioned above, KA neurotoxicity not only induces the generation of elevated levels of enzymic and nonenzymic lipid mediators of phospholipid metabolism but also increases the synthesis of ceramide, which may induce a spontaneous formation of large ceramide-enriched membrane platforms. These large ceramide-enriched membrane platforms may facilitate the increased production of ROS [45]. In neural membranes, both ceramide and its metabolites (ceramide-1-phosphate and sphingosine-1-phosphate) stimulate the generation of ARA, ARA-derived lipid mediators, and ROS through the stimulation of PLA₂ isoforms and arachidonic acid cascade [5, 41, 46]. Similarly, ARA stimulates sphingomyelinase activity and generates more ceramide. Simultaneous intensification of these processes following KA-mediated toxicity not only increases the interplay (cross talk) among lipid mediators of phospholipid and sphingolipid metabolism but may also lower the levels of essential glycerophospholipid and ceramide molecular species in neural membranes, a process that may be closely associated with neurodegeneration [7]. Like ceramide-metabolizing enzymes, mechanisms associated with generation and release of ROS are also localized in membrane rafts. It is suggested that optimal integrity of ceramide-enriched rafts may be required for continuous cellular ROS release during KA-mediated neurotoxicity [7, 47]. Furthermore, KA stimulates the hydrolysis of plasmalogens, the vinyl ether-containing glycerophospholipids, by plasmalogen-selective-PLA₂ (PlsEtn-PLA₂) in a dose- and time-dependent manner [48], and ceramide decreases the levels of plasmalogens by inhibiting PlsEtn-PLA₂ in rat brain slices [49]. The decrease in plasmalogen levels by ceramide can be blocked by quinacrine, ganglioside, and bromoenol lactone. These compounds inhibit PlsEtn-PLA₂ activity. Thus it is likely that interplay between plasmalogen and sphingomyelin-derived lipid mediators may modulate inflammation and oxidative stress. These processes are closely associated with KA neurotoxicity [48, 49]. Collective evidence suggests that high levels of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators in KA-mediated neurotoxicity may disturb normal signal transduction homeostasis and threaten neural cell survival due to increased intensity of cross talk among glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators [7].

4.6 KA-INDUCED NEUROTOXICITY AND ITS IMPLICATION FOR NEUROTRAUMATIC AND NEURODEGENERATIVE DISEASES

Involvement of excitotoxicity, oxidative stress, and neuroinflammation in neurotraumatic [stroke, spinal cord injury (SCI), and traumatic brain injury (TBI)] and

neurodegenerative diseases [AD, Parkinson disease (PD), and amyotrophic lateral sclerosis (ALS)] has gained increasing acceptance, but the underlying mechanisms through which excitotoxicity, oxidative stress, and neuroinflammation mediate neurodegeneration still remain elusive [50]. In addition, recent data from human studies highlight the association of KA-Rs in certain psychiatric diseases, such as schizophrenia and major depression, and a recent association of KA-R gene variants with response to antidepressants has brought considerable interest in developing a clearer understanding of KA-R action in the brain [51]. It is reported that exposure to stress and stress hormone administration can produce contrasting changes in KA-R subunit expression in the rat hippocampus, suggesting that a modification of hippocampal KA-Rs by stress may be a mechanism for predisposing individuals to stress-related psychiatric diseases [51]. Enhanced rate of interplay (cross talk) among excitotoxicity, oxidative stress, and neuroinflammation through lipid mediators may be associated with the increased vulnerability of neurons in neurotraumatic, neurodegenerative, and neuropsychiatric diseases [5, 46].

KA-induced neurotoxicity, excessive activation of KA type of glutamate receptors leads to a number of deleterious consequences, including depolarization-mediated increase in Ca²⁺ influx, stimulation of Ca²⁺-dependent enzymes (PLA₂ isoforms, NOS, calpains, caspases, and protein kinases), increased expression of proinflammatory cytokines, depletion of ATP, increase in lipid peroxidation products (4-HNE, isoprostanes, ROS), activation of the mitochondrial permeability transition, and loss of glutathione [5, 7, 14]. Like KA-induced neurotoxicity, neurodegeneration in neurotraumatic diseases is accompanied by enhanced degradation of neural membrane glycerophospholipid, activation of PLA₂, NOS, calpains, caspases, and protein kinases, increased expression of cytokines, and production of ROS and lipid hydroperoxides. Generation of 4-HNE, isoprostanes, and ROS induces oxidative stress, whereas formation of high levels of prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor contributes to neuroinflammation [5, 46, 50, 52]. A major source for vascular and neuronal ROS is a family of nonphagocytic NADPH oxidases, including the prototypic Nox2 homolog-based NADPH oxidase, as well as other NADPH oxidases, such as Nox1 and Nox4 [53]. Other possible sources include mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled NOS. NADPH oxidase-derived ROS plays a physiological role in the regulation of neural and endothelial function. Although the pathophysiological importance of glutamate-mediated glycerophospholipid degradation in neurotraumatic diseases is

not fully understood, it is proposed that enhanced catabolism of glycerophospholipids in neurotraumatic diseases may be an earliest event [46].

As stated above, neurodegeneration in KA-induced neurotoxicity is accompanied by excitotoxicity-mediated enhanced degradation of neural membrane glycerophospholipid, activation of PLA₂, NOS, calpains, caspases, and protein kinases, increased expression of cytokines, and production of 4-HNE, isoprostanes, and ROS and lipid hydroperoxides [5, 46, 50, 52]. In contrast, neurodegenerative diseases are accompanied by neurodegeneration caused by many different factors, including but not limited to genetic abnormalities, accumulation of abnormal extracellular and intracellular deposits (β -amyloid, α -synuclein, huntingtin, etc.) in specific populations of neurons in specific areas of the brain, changes in neural membrane composition due to increase in activities of PLA₂, COX-2, iNOS, caspases, and calpains, neurotransmitters, and their receptors, alterations in cerebral blood flow and blood-brain barrier, and problems in the immune system [52]. The most important risk factors for sporadic neurodegenerative diseases are old age, positive family history, unhealthy lifestyle, endogenous factors, and exposure to a toxic environment [5, 46, 54]. Neurodegenerative diseases commence late in life and are accompanied by the loss of synapses and accumulation of misfolded protein aggregates [5, 52]. The chemical nature of the misfolded protein aggregate is different in each neurodegenerative disease. For example, β -amyloid peptide and Tau protein aggregate and accumulate in plaques and tangles of AD patients; α -synuclein and perkin accumulate in Lewy bodies of PD patients, huntingtin accumulates as nuclear inclusions in Huntington disease (HD) patients, and mutation in Cu/Zn superoxide dismutase occurs in some inherited forms of ALS. In some neurodegenerative diseases alterations in glutamate homeostasis may also contribute to neurodegeneration. For example, in AD levels of glutamate are not altered, but a marked reduction in the expression of NR2A and NR2B subunit mRNA has been reported in the hippocampus and entorhinal cortex in the brain of AD patients. In addition, alterations in activities of glutamate transporters have been observed in the brain of AD patients [55]. This may induce changes in glutamate homeostasis in AD, causing a major disturbance in Ca²⁺ homeostasis [7] and inducing neurochemical changes similar, but not identical, to KA-induced neurotoxicity. Similarly, involvement of excitotoxicity in the pathogenesis of ALS is supported by the decrease in RNA editing of AMPA receptor subunit GluR2 at the Q/R site in the motor neurons and reduction in glial glutamate transporter GLT-1 in brain tissue from sporadic ALS patients [56–58]. Involvement of the NMDA-type of glutamate

receptors in pathogenesis of HD in animal models has also been reported [59].

In neurotraumatic diseases, because of the faster rate of upregulation of interplay among excitotoxicity, oxidative stress, and neuroinflammation, neurons die rapidly in a matter of hours to days following the sudden lack of oxygen, decreased ATP level, and sudden collapse of ion gradients. In contrast, in neurodegenerative diseases, oxygen, nutrients, and ATP continue to be available to the nerve cells and ionic homeostasis is maintained to a limited extent. The interplay among excitotoxicity, oxidative stress, and neuroinflammation occurs at a slow rate, resulting in a neurodegenerative process that takes several years to develop [7, 46, 52].

4.7 CONCLUSION

Neural membranes are composed of glycerophospholipids, sphingolipids, cholesterol, and proteins. KA-mediated toxicity causes enrichment of glycerophospholipid, sphingolipid, and cholesterol metabolism that results in elevations of glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators. The increased interaction among glycerophospholipid-, sphingolipid-, and cholesterol-derived lipid mediators intensifies degradation of neural membrane components and loss of essential glycerophospholipids, sphingolipids, and cholesterol, causing changes in neural membrane fluidity and permeability. These processes allow a sustained Ca²⁺ influx and produces stimulation of Ca²⁺-dependent enzymes including PLA₂, PLC, PLD, NOS, calpains, and endonucleases resulting in stimulation of lipolysis and proteolysis, production of lipid peroxides, and loss of glutathione. Stimulation of these enzymes along with mitochondrial dysfunction, alteration in cellular redox, induction of cytokines, and decrease in ATP levels may be responsible for neural cell death in KA-mediated neurotoxicity.

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SURVIVAL STRATEGY AND DISEASE PATHOGENESIS ACCORDING TO THE Nrf2-SMALL Maf HETERODIMER

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5.1 INTRODUCTION

Organisms, including mammals, are constantly exposed to oxidative stress due to oxygen consumption during aerobic respiration and xenobiotic chemicals in their diet and/or the environment. Mammalian cells are equipped with sophisticated machinery for cell protection against oxidative and xenobiotic stress. NF-E2-related factor 2 (Nrf2) is a potent transcription activator that plays a central role in regulating the expression of genes encoding detoxifying enzymes and antioxidant proteins by binding to the antioxidant response element (ARE)/electrophile response element (EpRE). Nrf2 is a member of the Cap'n'Collar (CNC) transcription factor family that commonly contains a unique stretch of amino acids, designated the CNC domain, followed by a well-conserved basic region-leucine zipper (bZIP) motif. Under unstressed conditions, Nrf2 is ubiquitinated by kelch-like ECH-associated protein 1 (Keap1) and degraded by the proteasome in the cytoplasm. Upon exposure to oxidative or xenobiotic stress, Nrf2 is stabilized, translocates into the nucleus, heterodimerizes with small Maf, another bZIP protein, binds to the ARE/EpRE, and activates transcription. Recent studies have clarified the intricate molecular mechanisms of Nrf2 activation in response to stress and revealed the involvement of Nrf2 in many human diseases including neurodegeneration, airway disorders, cardiovascular disease, and cancer. This review covers the historical aspects of the discovery of Nrf2,

recent advances in molecular studies of Nrf2 function, updated reports on the involvement of Nrf2 in various pathological conditions, and perspectives of Nrf2 utilization for human welfare.

5.1.1 Identification of Nrf2

The antioxidant response element (ARE), also known as an electrophile response element (EpRE) [TGA(G/C)NNNGC], was first identified in analyses of induction mechanisms of the detoxifying enzyme genes in response to electrophilic chemicals [1–3]. ARE/EpREs are often found in the promoter regions of cytoprotective genes and are considered to be critical for the inducible expression of these genes. Although the importance of this *cis*-regulatory element was clearly shown, the *trans*-acting factor responsible for inducible transcription activation remained unknown.

During the search for the *trans*-acting factor, an interesting coincidence occurred in research on transcriptional regulation. Nuclear factor erythroid 2 (NF-E2) is a transcriptional activator important for erythroid-specific gene expression through interaction with NF-E2 binding sites [ATGA(G/C)TCAGCA] [4, 5]. Nrf2 was originally identified as a homolog of NF-E2 and was found to associate with the NF-E2 binding site as expected [6, 7] (Fig. 5.1). Similarities between the ARE/EpRE sequence and the NF-E2 binding site led to the discovery of Nrf2 as the ARE/EpRE binding factor [8] (Fig. 5.2A).

Nrf2 possesses a characteristic CNC domain followed by a well-conserved bZIP motif (Fig. 5.1). The CNC domain was defined based on the sequence homology of 43 amino acids to the *Drosophila* Cnc protein [9]. In addition to NF-E2 and Nrf2, four additional factors were isolated as members of the CNC family: Nrf1, Nrf3, Bach1, and Bach2 [10, 11, 12]. NF-E2, Nrf1, Nrf2, and Nrf3 are considered to be activators [4, 6, 7, 10, 11], while Bach1 and Bach2 possess characteristic Broad complex-Tramtrack-Bric-a-brac (BTB) domains at their N-terminal ends and are considered to be repressors [12–14] (Fig. 5.2B). Importantly, none of the members of the CNC family binds efficiently to DNA as monomer or homodimer, and each requires small Maf, another bZIP protein, as an obligate heterodimeric partner molecule [15] (Fig. 5.1 and Fig. 5.2B).

The CNC protein homolog in *Caenorhabditis elegans* is SKN-1, and it binds to DNA as a monomer because it does not possess the leucine zipper structure [16]. The SKN-1 recognition sequence is similar to half-sites of ARE/EpRE and NF-E2 binding site (Fig. 5.2A). One

side of the site is recognized by CNC proteins, and small Maf proteins recognize the other side of the site, which contains a GC dinucleotide. Nrf2 shares a heterodimeric partner molecule with other CNC proteins, and all of the CNC-small Maf heterodimers recognize a nearly identical DNA binding sequence. This implicates cross talk between Nrf2 and other members of the CNC and small Maf transcription factor families.

5.1.2 Susceptibility of *Nrf2*-Null Mice to Oxidative and Xenobiotic Stress

Analysis of *Nrf2*-null mice clearly demonstrated the substantial contribution of Nrf2 to the inducible regulation of cytoprotective genes [8]. *Nrf2*-null mice display an increased formation of DNA adducts in the lung following exposure to diesel exhaust [17], a more severe liver toxicity after administration of acetaminophen [18], an increased susceptibility to cigarette smoke-induced emphysema [19], and an aggravated bleomycin-induced pulmonary fibrosis

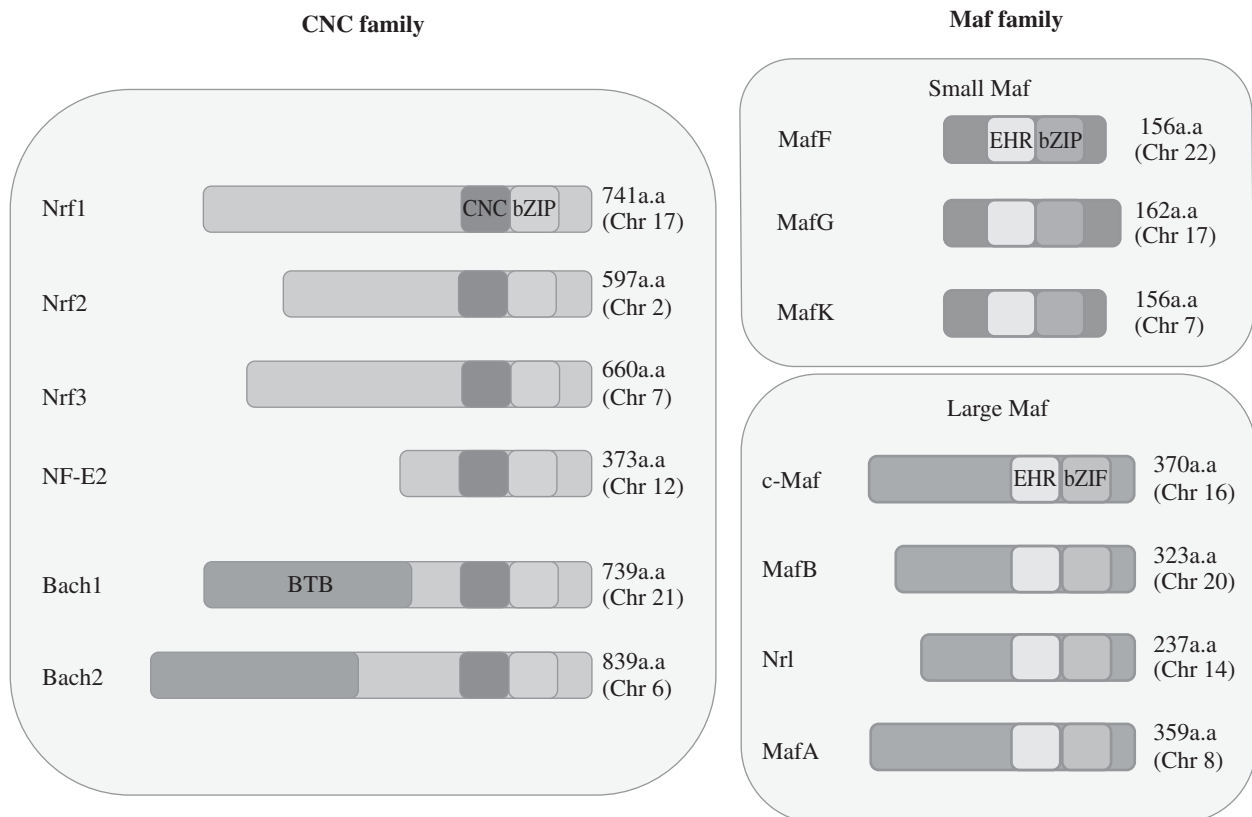


Fig. 5.1 The structures of CNC and Maf family proteins. The CNC family contains four transcriptional activators, Nrf1, Nrf2, Nrf3, and NF-E2, and two transcriptional repressors, Bach1 and Bach2. The Maf family consists of three small Maf transcription factors and four large Maf transcription factors. The bZIP motifs are a common structural feature of CNC and Maf family proteins. The CNC and EHR domains are unique to CNC and Maf family proteins, respectively. Bach1 and Bach2 possess BTB domains in their N-terminal regions. The amino acid number and chromosomal location of the human gene encoding each protein are shown.

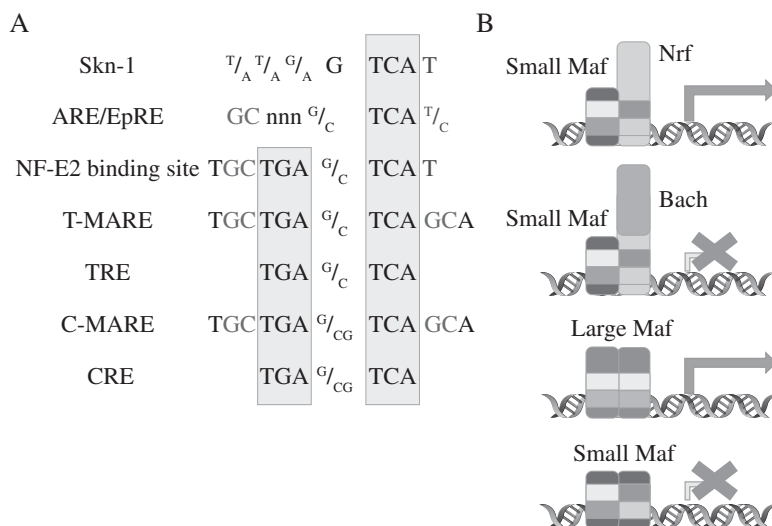


Fig. 5.2 DNA recognition sequences and the transcriptional activities of Maf-containing dimers. (A) Various MARE-related sequences are shown. The dinucleotide “GC” (marked in red) is essential for recognition by Maf proteins. The nucleotide “T” or “T/C” (marked in blue) enhances the binding of CNC proteins. Trinucleotides (boxed in gray) with the central G and GC sequence consist of the TRE and CRE, respectively, which make up the core region of the MARE. (B) Nrf1, Nrf2, Nrf3, and NF-E2 activate transcription by forming heterodimers with small Maf, while Bach1 and Bach2 repress transcription. A large Maf homodimer, possessing a *trans*-activation domain, activates transcription, while a small Maf homodimer, lacking a *trans*-activation domain, represses transcription. All of these Maf-containing dimers bind to T-MARE with high affinity. (See color insert.)

[20]. Nrf2 confers resistance to xenobiotic stress through activation of the genes encoding detoxifying enzymes, including glutathione *S*-transferases (GST), UDP glucuronate transferases (UGT), and NAD(P)H:quinone oxidoreductase 1 (NQO1) [8, 21–23]. The phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults are summarized in Table 5.1.

Nrf2 also plays an important role in response to oxidative stress by activating antioxidant proteins and enzymes for glutathione synthesis [21, 24]. Cellular capacities for elimination of reactive oxygen species (ROS) are limited in the absence of Nrf2 [25]. Nrf2 regulates intracellular ROS levels not only in pathological conditions but also during the physiological cellular differentiation process of megakaryocytic maturation [26]. With decreased capacities to eliminate ROS of endogenous origins, *Nrf2*-null mice tend to spontaneously develop various inflammatory disorders, including glomerulonephritis, immune-mediated hemolytic anemia, and multiorgan autoimmune inflammation [27–29], all of which appear to be due to a chronic increase in ROS. The phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults are summarized in Table 5.2.

The *Nrf2* gene is ubiquitously expressed in various cell lineages and at both the embryonic and adult stages. The expression level is relatively higher in the lung, kidney, and liver in adults and in the intestine, lung, and choroid plexus in embryos [30]. Chicken Nrf2 is highly expressed in the kidney and intestine [7].

The expression level of the *Nrf2* gene is regulated by a positive feedback mechanism dependent on an ARE/EpRE in the promoter region of the *Nrf2* gene [31]. The *Nrf2* gene is also transcriptionally induced by the aryl hydrocarbon receptor [32].

Analysis of a single nucleotide polymorphism (SNP) in the promoter region of the *Nrf2* gene demonstrated the significance of the transcriptional regulation of the *Nrf2* gene in the determination of Nrf2 activity. A SNP in the promoter region of the mouse *Nrf2* gene is linked to the reduced expression of the *Nrf2* gene and susceptibility to hyperoxic lung injury in the C57BL/6J mouse strain [33]. Indeed, exposure to hyperoxia causes more severe lung damage in *Nrf2*-null mice than in wild-type mice [34]. SNPs found in the promoter region of the human *NRF2* gene [35] are linked to a higher risk of acute lung injury [36]. These data demonstrate the importance of transcriptional regulation of the *Nrf2* gene in stress response.

5.2 THE KEAP1-NRF2 SYSTEM IN RESPONSE TO ELECTROPHILES

Electrophiles have substantial impacts on biomolecules including proteins and nucleic acids that are rich in electron-dense parts. An atom with stronger electronegativity, such as oxygen, attracts pi electrons, and consequently an electron-deficient part is generated, which

TABLE 5.1 Phenotypes observed in *Nrf2*-null mice after exposure to exogenous insults

Organ	Reagent	Phenotype(s)	Inflammation-related genes affected in <i>Nrf2</i> -null mice	Reference
Skin	DMBA/TPA	Increased incidence of skin tumors and tumor numbers		119
	Diesel exhaust	Severe epithelial hyperplasia and increased levels of 8-OHdG in the lung		17
	Benzo[a]pyrene	Increased somatic mutations of the <i>Gpt</i> gene in lungs		121
Gastric	DSS	Susceptibility to DSS-induced colitis	IL-1 β , IL-6, iNOS, COX-2, TNF- α	156
	AOM/DSS	Susceptibility to AOM/DSS-induced colitis	TNF- α , IL-1 β , IL-12p40	157
	Benzo[a]pyrene	Increased tumor numbers in the stomach		65, 158, 159
Liver	AOM/DSS	Increased incidence of colonic tumors	COX-2, 5-LOX	122
	IQ	Increased incidence of liver tumors and tumor numbers		160
	D3T, CDDO-Im	Reduced expression of detoxifying genes and antioxidant genes		148, 161
Bladder	Acetaminophen	Acute hepatotoxicity		18
	BBN	Increased incidence of bladder tumors		23
Brain	LPS	Increased microglial infiltration	iNOS, IL-6, TNF- α	16
Lung	LPS, sepsis induced by cecal ligation and puncture	Exacerbated lung inflammation	TNF- α , enhanced activation of NF- κ B	163
	Carrageenin	Exacerbated lung inflammation, increased neutrophil infiltration		133
	Ovalbumin sensitization and challenge	Exacerbated allergen-driven asthmatic inflammation, hyperresponsiveness to cholinergic challenge	IL-4, IL-13, enhanced activation of NF- κ B	164
	Cigarette smoke	Susceptibility to cigarette smoke-induced emphysema	Activation of caspase-3, decreased SLPI	19, 134
	Elastase	Susceptibility to elastase-induced lung inflammation and emphysema	Decreased SLPI	135

DMBA/TPA, 7,12-Dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate;

DSS, dextran sulfate sodium; AOM, azoxymethane; IQ, 2-amino-3-methylimidazo

[4,5-*f*]quinoline; D3T, 3*H*-1,2-dithiole-3-thione; CDDO-Im, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole; BBN, *N*-nitrosobutyl(4-hydroxybutyl)amine; LPS, lipopolysaccharide; SLPI, secretory leukoprotease inhibitor.

TABLE 5.2 Phenotypes observed in *Nrf2*-null mice that develop spontaneously or in response to endogenous insults

Organ	Phenotype(s)	Reference
Skin	Prolonged inflammation	165
Brain	Astrogliosis and vacuolar leukoencephalopathy after 1 year of age	166
	Exacerbated leukocyte infiltration, increased infarct size, and severe behavioral deficits after the intracerebral hemorrhage	167, 168
Immune system	Autoimmune inflammation and lymphoproliferation in multiple organs	29
	Lupus-like autoimmune nephritis	27
Hematopoietic	Immune-mediated hemolytic anemia	28
Craniofacial	De-colorization of incisors	169

attacks electron-dense parts of nucleic acids and proteins. The protein adduct leads to acute toxicity due to cellular dysfunction, and the DNA adduct formation leads to carcinogenesis. The Keap1-Nrf2 system is a

mechanism of cytoprotection from such electrophilic insults. The extremely sensitive nature of several thiol residues of Keap1 to electrophiles enables the system to respond to the stimuli in the first place.

5.2.1 Characteristic Features of Nrf2 Inducers

The most important feature of gene expression regulated by Nrf2 is inducibility upon exposure to xenobiotic and oxidative stress (Fig. 5.3). Nrf2 is a short-lived protein with a half-life of approximately 20 min [37, 38]. Proteasomal inhibitors dramatically increase the amount of Nrf2 protein, indicating that Nrf2 is constantly degraded by the proteasome. While Nrf2 is hardly detected during unstressed conditions, the half-life of Nrf2 becomes longer, allowing easy detection of Nrf2 in nuclei, during stressed conditions [37, 39]. Xenobiotic and oxidative stress inhibit proteasome-dependent degradation of Nrf2. Stabilization of Nrf2 upon exposure to stress is critical for the inducible expression of many cytoprotective genes.

A common feature of the diverse substances that act on Nrf2 is electrophilicity (Fig. 5.4). Typical Nrf2 inducers are diethyl maleate (DEM), *tert*-butylhydroquinone (tBHQ), and sulforaphane (SFN), all of which are electrophilic. Many other electrophiles, including ebselen (Ebs) and 8-nitroguanosine 3', 5'-cyclic monophosphate (8-nitro-cGMP), have been shown to activate Nrf2 [40, 41]. Thus electrophiles can stabilize Nrf2, which allows Nrf2 to accumulate in nuclei and activate transcription.

5.2.2 Identification of Keap1

An important finding that led to the revelation of the molecular mechanism for how Nrf2 is stabilized by electrophiles originated from an analysis of the N-terminal region of Nrf2. Deletion of the N-terminal region of Nrf2 markedly enhances its transcriptional activity [42]. The N-terminal region corresponds to Nrf2-ECH homology 2 (Neh2), which is a highly homologous domain between human and chicken Nrf2. Nrf2 is divided into six domains, ranging from Neh1 to Neh6 [42] (Fig. 5.5A). Neh1 includes a bZIP motif and is involved in DNA binding, heterodimerization, and nuclear translocation. Neh4 and Neh5 are essential for strong transcriptional activity upon coactivator binding, such as CBP [43]. The Neh2 domain negatively regulates Nrf2 transcriptional activity.

The Kelch-like ECH associated protein 1 (Keap1) was identified as a novel cytoplasmic factor that interacts with the Neh2 domain of Nrf2 [42]. Keap1 is divided into three main parts (Fig. 5.5B). The BTB domain resides in the N-terminus, while the DC domain, which includes the double glycine repeat (DGR) and the carboxyl-terminal region (CTR), resides in the C-terminus. The region between the BTB and DC domains is called the intervening region (IVR). Molecular dissection of Nrf2 and Keap1 demonstrated that the Neh2 domain of Nrf2

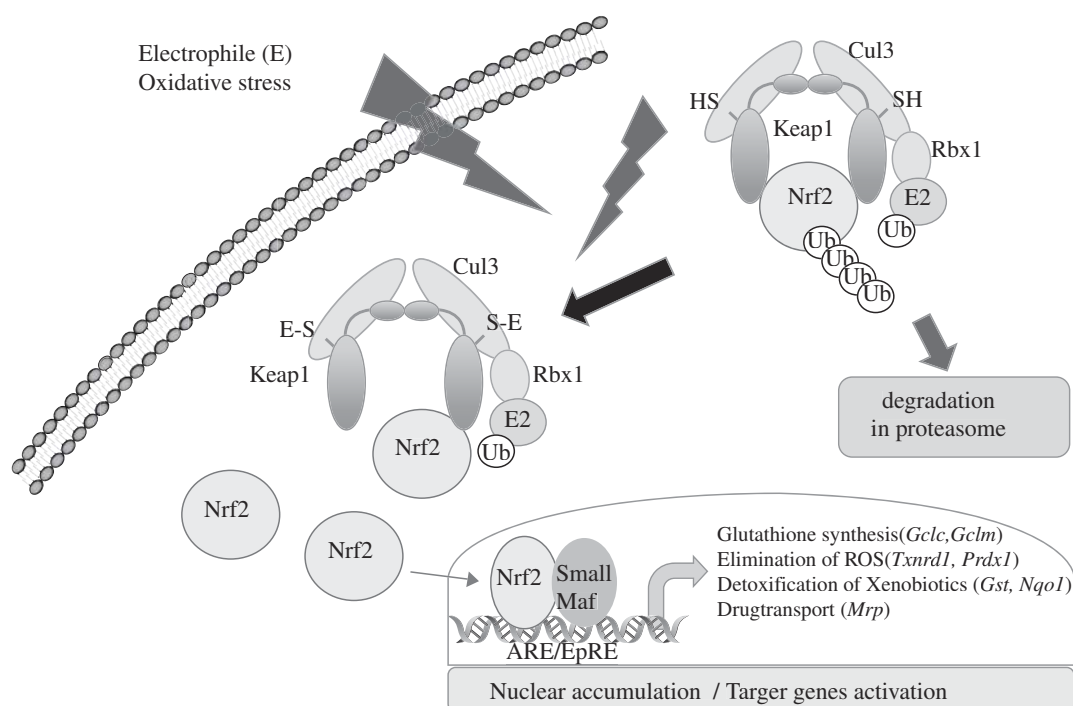


Fig. 5.3 The mechanism of Nrf2 activation in response to electrophiles and oxidative stress. In unstressed conditions, Nrf2 shows a rapid turnover due to ubiquitination and degradation via the proteasome. The Keap1-Cul3 complex serves as an E3 ubiquitin ligase for Nrf2. On exposure to electrophiles or oxidative stress, reactive thiols of Keap1 (-SH) are modified (-SE), resulting in a decline in enzymatic activity. Nrf2 is stabilized, and de novo synthesized Nrf2 translocates into nuclei, heterodimerizes with small Maf, and activates target genes encoding antioxidant proteins, detoxifying enzymes, and other cytoprotective proteins.

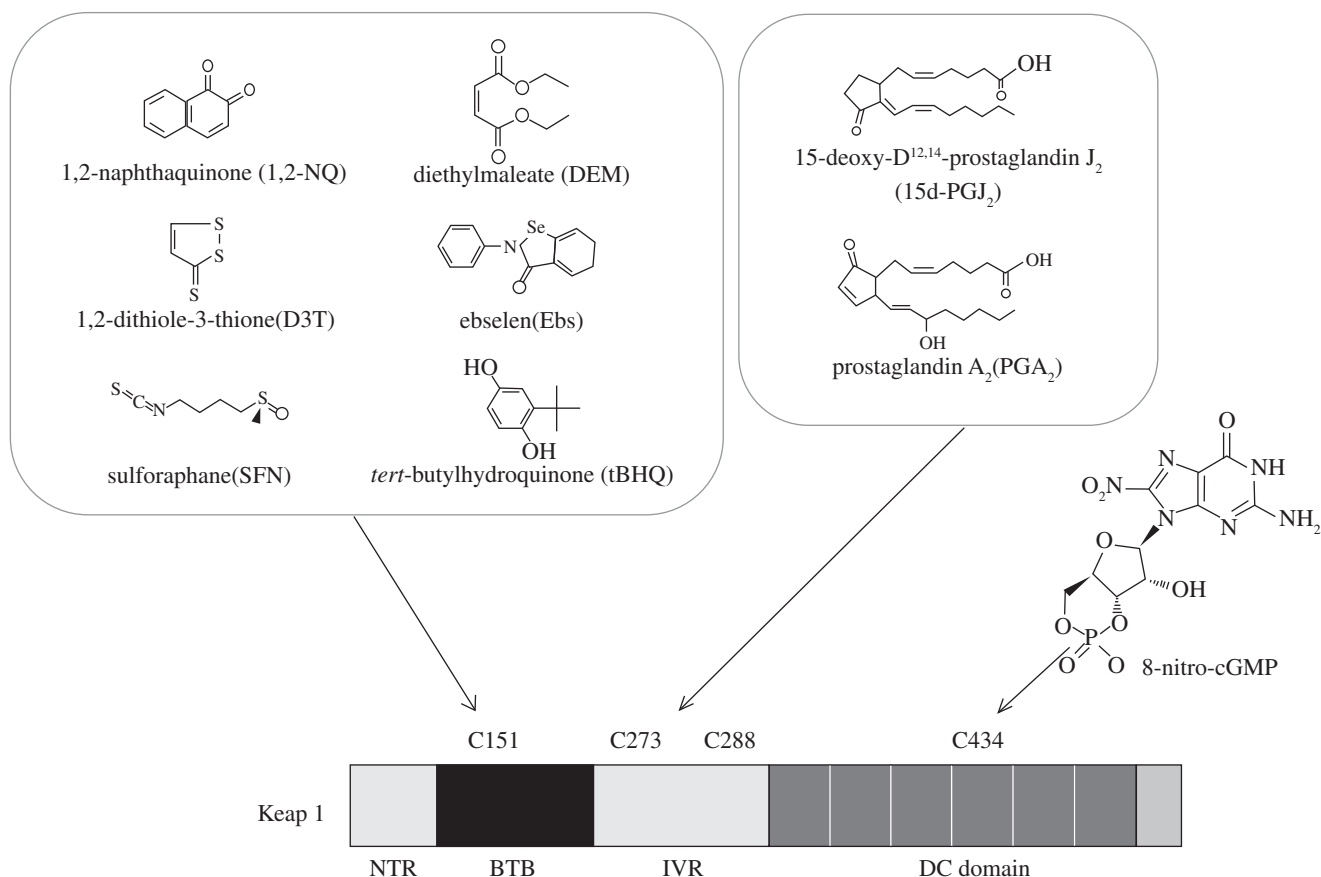


Fig. 5.4 Electrophile-induced Nrf2 activity and the Keap1 sensor system. Distinct cysteine residues are involved in sensing different types of electrophiles. Cys-151 is necessary for Keap1 to respond to a group of electrophiles. Bulky electrophiles, including 15d-PGJ₂ and PGA₂, are considered to target Cys-273 and Cys-288, while 8-nitro-cGMP modifies Cys-434.

interacts with the Keap1 DC domain [42]. An association between Keap1 and Nrf2 destabilizes Nrf2, inhibiting transcriptional activation of the stress response genes.

Keap1 is ubiquitously expressed in adult tissues, among which skin, the esophagus, and the forestomach display particularly abundant expression [44, 45]. An approximately 5.7-kbp region upstream of the mouse *Keap1* gene harbors the enhancer activity for recapitulating *Keap1* expression profiles in vivo [45]. A genomic fragment containing this region was utilized for expressing Keap1 or its mutant molecule in transgenic mice, which were then bred into the *Keap1*-null background to evaluate the function of the Keap1 mutant molecule in the *Keap1*-null background (see Section 5.2.5).

5.2.3 Nrf2 is the Most Substantial Target of Keap1

Disruption of the *Keap1* gene in mice revealed that Nrf2 is actually stabilized in the absence of Keap1. Subsequently, Nrf2 localizes to nuclei and activates target genes for cytoprotection. *Keap1*-null cells, which

constitutively express high levels of cytoprotective genes, were expected to be resistant to xenobiotic and oxidative stress, and *Keap1*-null mice were expected to survive under severely stressed conditions. However, the phenotype of *Keap1*-null mice was unexpected. Instead of making mice more resistant to stress, *Keap1* deletion caused lethality 3 weeks after birth, and mice displayed severe hyperkeratosis of the esophagus and forestomach [44]. The lack of nutrition was considered to be the primary cause of the death, because keratinocyte-specific deletion of the *Keap1* gene results in the same preweaning lethality [46]. Mice can survive to adulthood if they escape this feeding problem [47].

An intriguing notion emerged from the analysis of mice harboring the hypomorphic allele of *Keap1* (*Keap1*^{fllox}), in which the effect of the graded expression of Keap1 in vivo was examined [46]. Insertion of two loxP sequences into the *Keap1* locus for conditional disruption of the *Keap1* gene actually reduced expression of *Keap1*. *Keap1*^{fllox/-} mice displayed constitutive stabilization of Nrf2 in various tissues and were more resistant

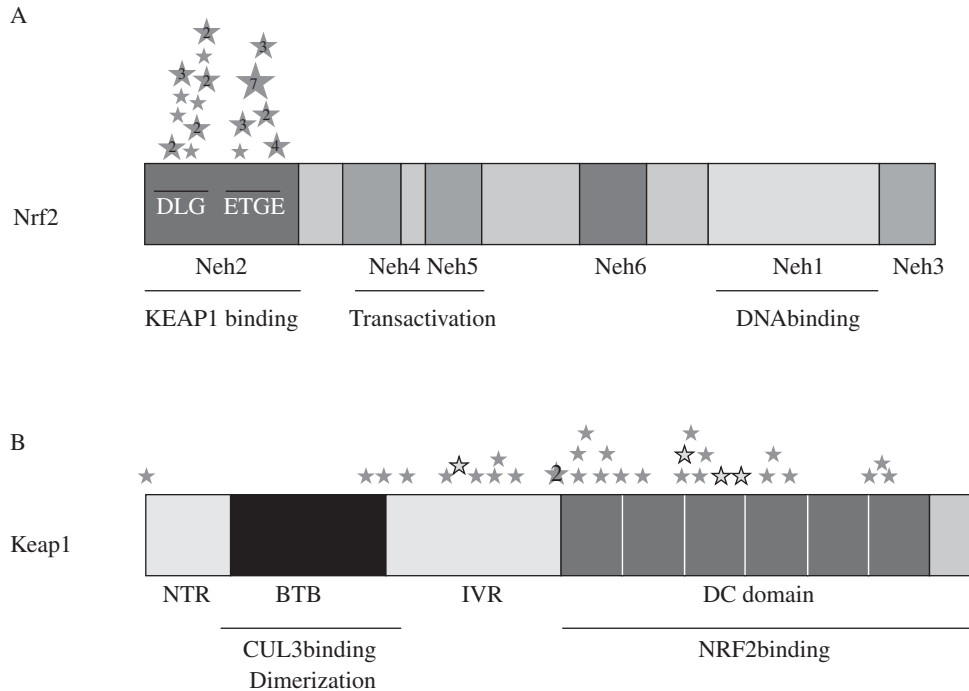


Fig. 5.5 The domain structures of Nrf2 and Keap1. (A) Nrf2 contains six well-conserved domains, Neh1 to Neh6. Neh2 is essential for binding to Keap1. Neh4 and Neh5 contribute to transactivation through interaction with CBP. Neh1 is involved in DNA binding and dimerization with small Mafs. Stars indicate the positions of amino acid substitution and deletion due to somatic mutations in the *NRF2* gene found in human cancers. When an amino acid is affected in more than two cases, the number of cases is shown in the star. All mutations are clustered within the DLG and ETGE motifs. The results of two reports are summarized [70, 71]. (B) Keap1 consists of a BTB domain, a DC (DLG and CTR) domain, and the IVR (between BTB and DC domain). The BTB domain contributes to homodimerization. BTB and IVR are involved in the interaction with Cul3. The DC domain is essential for the interaction with Nrf2. Stars without rims indicate the positions of amino acid substitution and deletion due to somatic mutations in the *KEAP1* gene found in human cancers. When an amino acid is affected in more than two cases, the number of cases is shown in the star. Stars with bold rims indicate the generation of stop codons due to somatic mutations, resulting in truncation of the KEAP1 protein. The results of six reports are summarized [68, 69, 72, 123, 124, 170].

to acetaminophen-induced liver toxicity than wild-type control mice. *Keap1*^{fllox/-}:Albumin-Cre mice, in which the *Keap1* gene is completely disrupted in liver, are not as resistant as hypomorphic *Keap1*^{fllox/-} mice. Thus the inducible and transient activation of Nrf2 is beneficial for cytoprotection, while constant and intense activation of Nrf2 seems to be disadvantageous [48].

Importantly, simultaneous disruption of the *Nrf2* gene completely rescued lethality in *Keap1*-null pups [44], suggesting that constitutive activation of Nrf2 is the cause of the death and that Nrf2 is the most substantial target of Keap1.

5.2.4 The Keap1-Nrf2 Complex for Nrf2 Degradation

Keap1 is responsible for the degradation of Nrf2 in unstressed conditions. Further studies revealed that Keap1 is an adaptor molecule of the Cullin 3-based ubiquitin E3 ligase [38, 49–51] (Fig. 5.3). Ubiquitination of Nrf2 is inhibited in the absence of Keap1, and,

consequently, Nrf2 is stabilized and accumulates in nuclei. In the absence of stimuli, low expression levels of Nrf2-dependent genes are maintained by the ubiquitin E3 ligase activity of the Keap1-Cul3 complex.

Structural and biochemical analyses revealed the molecular mechanisms of Nrf2 degradation. Keap1 homodimerizes through its BTB domain [52], and the overall structure of the homodimer resembles a “cherry-bob” [53] (Fig. 5.6A). The global structure of the cherry-bob is composed of a DC domain, the IVR and part of the BTB domain. A pair of Keap1 homodimers possesses two DC domains, in which one molecule of Nrf2 associates using two discrete motifs within the Neh2 domain, the ETGE and DLG motifs [54] (Fig. 5.6B). The two-site binding of Keap1 and Nrf2 seems to be beneficial for the ubiquitination and subsequent degradation of Nrf2 because seven lysine residues, which are targets of ubiquitination, are aligned at the same surface of the α -helix structure between the ETGE and DLG motifs.

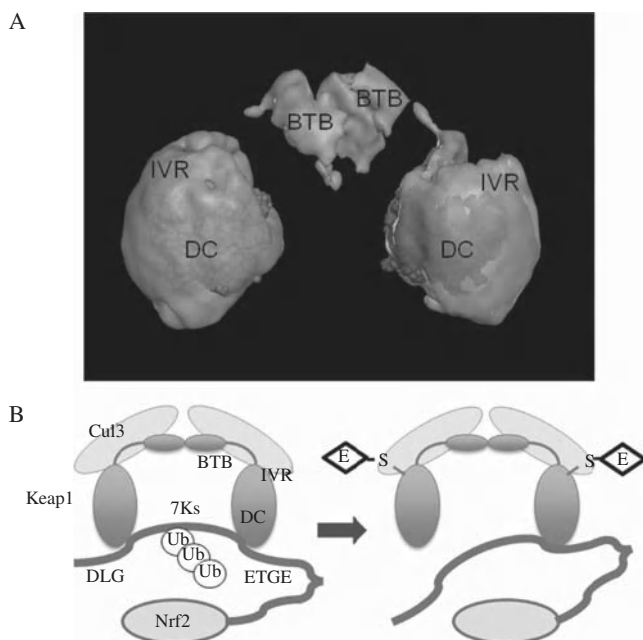


Fig. 5.6 The overall structure of the Keap1 homodimer and the regulation of Nrf2 activity by Keap1. (A) The three-dimensional structure of the Keap1 homodimer (cited from Ogura et al., *Proc Natl Acad Sci USA*. 2010;2842-7, 107). (B) Interaction between Nrf2 and the Keap1-Cul3 complex under unstressed conditions (left) and stressed conditions in which thiols are modified with electrophiles (E) (right). Each DC domain of the Keap1 homodimer binds to the DLG and ETGE motifs in the Neh2 domain of Nrf2. Keap1 is proposed to interact with Cul3 at the BTB domain and the IVR. Nrf2 is polyubiquitinated at 7 lysine residues (7Ks) between the DLG and ETGE motifs (left). Modification of Keap1 with electrophiles is thought to alter the overall conformation of the Keap1-Cul3 and Nrf2 complex, which inhibits Nrf2 ubiquitination (right). (See color insert.)

5.2.5 Keap1 as a Sensor Molecule for Electrophiles

Upon exposure to xenobiotic and oxidative stress, Keap1 stops the ubiquitination of Nrf2. Stabilized Nrf2 translocates into nuclei, heterodimerizes with small Maf, and activates the transcription of target genes (Fig. 5.3). An important question is how the stimuli repress the E3 ligase activity of the Keap1-Cul3 complex.

Keap1 is a thiol-rich protein and possesses many reactive cysteine residues neighboring basic amino acids. Covalent binding of electrophiles to cysteine residues has been observed *in vitro* and *in vivo* by mass spectrometry [55, 56]. The significance of the cysteine residues to Keap1 function was demonstrated *in transfecto* [57, 58] and *in vivo* [45]. The contributions of Cys-151 in the BTB domain and Cys-273 and Cys-288 in the IVR to Keap1 function were the focus of the *in vivo* studies. Transgenic mice expressing a mutant Keap1 under the regulation of the endogenous *Keap1* gene regulatory region were generated and crossed into the *Keap1*-null background.

The *in vivo* contributions of each cysteine residue to Keap1 function was evaluated by this transgenic complementation rescue method. Alanine substitution of Cys-273 and Cys-288 abrogated the repressor function of Keap1, while serine substitution of Cys-151 did not affect the repressor function but impaired the responsiveness of Keap1 to a group of electrophiles [45] (Fig. 5.4). Thus Cys-273 and Cys-288 are essential for Keap1 to repress Nrf2 activity in unstressed conditions, while Cys-151 is essential for the response to electrophilic stimuli.

A more detailed analysis revealed that Keap1 senses various types of electrophiles utilizing distinct cysteine residues [59, 60] (Fig. 5.4). Many of the typical electrophiles, including DEM, tBHQ, SFN, Ebs, D3T, and 1,2-NQ, are sensed by Cys-151. Bulky electrophiles such as 15d-PGJ₂ and PGA₂ seem to attack Cys-273 and Cys-288. Cys-434 is involved in nitric oxide signaling [61]. Cys-226 and Cys-613, in addition to His-225, are critical for sensing metal ions such as Cd²⁺ and Zn²⁺ [60]. These results indicate that Keap1 is a multifaceted electrophile sensor. Diverse inputs to Keap1, which are sensed in multiple ways using discrete cysteine residues, are processed to a single output, namely, inhibition of Nrf2 ubiquitination and subsequent Nrf2 stabilization.

Keap1 possesses multiple thiols with a lower pK_a, as 10 μM 8-nitro-cGMP, one of the endogenous electrophiles generated downstream of nitric oxide, modifies Keap1 even in the presence of 10 mM glutathione [41]. This explains why Keap1 detects low electrophile concentrations within cells and serves as a sensitive sensor. Such sensitivity enables the Keap1-Nrf2 system to play a central role in the electrophile counterattack response for cell protection.

Modification of Keap1 alters the overall conformation of the Keap1-Nrf2 complex and/or alters the interaction between Keap1 and Cul3 [54, 62]. Because the affinity between the Neh2 DLG motif and the Keap1 DC domain is lower than that of the Neh2 ETGE motif and the Keap1 DC domain, modification of Keap1 appears to alter the conformation of the complex, which leads to dissociation of the weaker interaction between DLG and DC. The stronger interaction between ETGE and DC seems to be maintained, which is consistent with the finding that alanine substitution of Keap1 C273 and C288 abrogates its ability to degrade Nrf2 but retains its binding affinity to Nrf2 [63]. In addition, *de novo* synthesized Nrf2, and not Nrf2 dissociated from Keap1, translocates into the nucleus [63]. The “hinge and latch model” is one of the molecular mechanisms for Nrf2 stabilization following Keap1 modification [64] (Fig. 5.6B). Alternatively, Cul3 dissociates from Keap1 upon exposure to stimuli [62]. The electrophiles targeting Cys-151 seem to disrupt the Keap1-Cul3 interaction. Loss of the Cul3-Keap1 interaction results in a decline in Keap1-Cul3 E3 ligase activity.

5.2.6 Electrophile-Independent Activation of Nrf2

An analysis of *Nrf2*-null mice showed that the activation of Nrf2 by electrophiles is effective for the inhibition of carcinogenesis following a challenge with carcinogens [65] (Table 5.2). A field study in China proved that Nrf2 activation is potentially effective for cancer chemoprevention [66, 67]. Nrf2 activation is beneficial for reducing the risk of carcinogenesis because many detoxifying enzymes induced by Nrf2 promote the elimination of strong electrophiles that could easily attack nucleic acids and proteins to form carcinogenic adducts.

Therefore, it is surprising that Nrf2 is constitutively stabilized in several cancer cells independent of electrophilic stimuli. Somatic mutations of the *KEAP1* gene have been found in lung adenocarcinomas [68, 69], while somatic mutations in the *NRF2* gene have been mainly identified in squamous carcinomas of the lung [70] (Fig. 5.5). Since the early reports about lung cancers, somatic mutations of *KEAP1* and *NRF2* have been documented in various solid cancers [71–73]. Mutant *KEAP1* does not have the ability to ubiquitinate Nrf2, and, consequently, Nrf2 is constitutively stabilized. Intriguingly, *KEAP1* mutations often occur heterozygously, and heterozygous *KEAP1* mutations produce a dominant negative effect [68]. The cherry-bob structure and the two-site binding model offer a very rational explanation for this phenomenon [53, 74]. Keap1 exerts its function in the form of a dimer, and both Keap1 subunits need to be intact for the proper ubiquitination of Nrf2. A sophisticated study using a combination of *Keap1*-null mice and transgenic mice expressing wild-type and mutant Keap1 revealed that the heterodimer of wild-type Keap1 and mutant Keap1 is not functional as an adaptor for the E3 ubiquitin ligase [45]. Missense mutations in the *KEAP1* gene are one of the causes of the constitutive stabilization of Nrf2 (Fig. 5.5B).

Somatic mutations in the *NRF2* gene are another cause of the constitutive stabilization of Nrf2. Intriguingly, the DLG and ETGE motifs in the Neh2 domain, identified in the molecular dissection of Nrf2 protein, correspond to the hot spots of *NRF2* mutations in cancer cells [70]. Amino acid substitution in either the DLG or ETGE motifs results in inhibition of Nrf2 ubiquitination, which is consistent with the requirement for an intact two-site binding structure of the Keap1-Nrf2 complex for efficient ubiquitination of Nrf2 (Fig. 5.5A).

The third cause of the constitutive stabilization of Nrf2 is the repression of *KEAP1* gene expression by epigenetic mechanisms. DNA methylation of the *KEAP1* gene promoter has been observed frequently in several cancer cell lines [75, 76].

Although Keap1 and Nrf2 are functionally normal, several cases have been reported in which Nrf2 is

constitutively stabilized. The accumulation of certain proteins that disrupt the association between Keap1 and Nrf2 was found to be responsible for stabilizing Nrf2. One example of these proteins is p21^{Cip1/WAF1}. The C-terminal region of p21 associates with the DLG motif of Nrf2 [77], preventing the formation of the two-site Keap1-Nrf2 binding complex. Thus p21 facilitates Nrf2 stabilization and promotes the expression of cytoprotective genes, which explains the cytoprotective function of p21 [78]. Another example is p62, a polyubiquitin-binding protein, which targets various substrates for autophagy [79]. The STGE motif of p62 interacts with the Keap1 DC domain in a similar manner to that of the DLG motif of Nrf2 [80]. When autophagy is impaired, p62 accumulates and disrupts the formation of the two-site Keap1-Nrf2 binding complex, which leads to Nrf2 stabilization. Intriguingly, abnormal accumulation of p62 is often observed in various human pathological conditions [81, 82]. Whether Nrf2 makes any contributions, favorable or unfavorable, to this pathogenesis remains to be elucidated.

5.2.7 Posttranslational Modification of Nrf2

Several reports have demonstrated the posttranslational modification of Nrf2. Phosphorylation of Nrf2 has been shown to promote or repress Nrf2 activity depending on the context. Activation of protein kinase C (PKC) enhances Nrf2 activity and is accompanied by the phosphorylation of Nrf2 at Ser-40 [83, 84]. In contrast, GSK-3 β promotes nuclear exclusion of Nrf2 and the subsequent repression of the xenobiotic and antioxidant responses [85, 86]. Fyn kinase, activated by GSK-3 β , phosphorylates Nrf2 at Tyr-568, which induces nuclear export and degradation of Nrf2 and attenuates the expression of cytoprotective genes [87].

Nrf2 is also modified by acetylation. CBP/p300 directly binds and acetylates Nrf2 in response to arsenite-induced oxidative stress [88]. Lysine residues of the acetylation target reside within the Neh1 domain of Nrf2, which contains the bZIP motif for DNA binding and dimerization. Consistently, this modification compromised the DNA binding activity of Nrf2 in a promoter-specific manner. The Neh3 domain of Nrf2 was also shown to be acetylated, which affects the subcellular localization of Nrf2 [89].

5.3 NRF2 IN THE CNC-SMALL Maf TRANSCRIPTION FACTOR NETWORK

Stabilized Nrf2 due to the exposure to electrophilic stimuli translocates into the nucleus and forms a heterodimer with small Maf. Small Maf is an obligatory partner molecule of Nrf2, conferring DNA binding ability

and DNA recognition specificity. Functional deficiency of small Maf results in the summation of functional deficiency of each CNC protein. Because of the unique DNA recognition of small Maf, target genes regulated by CNC-small Maf heterodimer are distinct from those of other bZIP transcription factors including Jun and Fos families. Small Maf, being shared by all the CNC proteins, serves as a central node of the transcription factor network by CNC and small Maf proteins.

5.3.1 Small Maf Proteins as Heterodimerization Partners for Nrf2

Genetic evidence clarified the essential role of small Maf proteins as the heterodimeric partner molecules of CNC proteins, including Nrf2 [90–92]. Small Maf proteins comprise one of the subclasses of Maf family proteins. Maf proteins possess a unique bZIP motif accompanied by Maf-specific extended homology region (EHR) and bind to Maf recognition elements (MAREs; TGCTGA^{G(C)}/C_(G)TCAGCA) in the form of a homodimer [15] (Fig. 5.1 and Fig. 5.2). Large Maf proteins, for example, c-Maf, MafB, MafA, and NRL, additionally possess *trans*-activation domains in their N-terminal regions, whereas small Maf proteins, for example, MafG, MafK, and MafF, lack *trans*-activation domains. Small Maf homodimers thus repress transcription, while heterodimers composed of small Maf and CNC proteins with *trans*-activation domains activate transcription [93, 94]. CNC-small Maf heterodimers also bind to MAREs and related sequences including the ARE/EpRE and NF-E2 binding sites. A functional balance between the CNC and small Maf proteins may determine MARE-dependent transcription activity, ranging from repression to activation (Fig. 5.2B).

5.3.2 Small Maf Deficiency as the Summation of Each CNC Protein Deficiency

Because three small Maf proteins, MafF, MafG, and MafK, are highly homologous and functionally redundant, disruption of any single gene causes only a modest phenotype [90, 95, 96]. Double- or triple-mutant mice display more severe phenotypes, which are a composite of the phenotypes observed in mutant mice lacking each individual CNC protein. For example, small Maf-mutant mice display thrombocytopenia, neurodegeneration, and an impaired response to xenobiotic and oxidative stress [92, 97, 98]. The thrombocytopenia is characteristically observed in *NF-E2*-null mice [99]. The neurodegeneration is observed in neural tissue-specific *Nrf1*-null mice [100]. An impaired response to xenobiotic and oxidative stress is observed in *Nrf2*-null mice [8, 24]. Thus small Maf proteins are essential for CNC proteins to exert their activity as a transcription factor.

In addition to serving as a DNA-binding adaptor for the CNC proteins, small Maf plays additional roles in the regulation of the transcription activity of CNC proteins. Small Maf can be described as bidirectional in transcription, that is, it behaves as an activator or a repressor when it exists as a heterodimer with CNC or a homodimer, respectively (Fig. 5.2B). The latter function has been suggested to be dependent on the sumoylation of small Maf [101]. The former function requires proper subnuclear localization of the CNC-small Maf heterodimer, which is directed by the C-terminal region of small Maf [102]. Interestingly, the lysine residue for sumoylation and the C-terminal region for appropriate subnuclear targeting both reside outside of the bZIP motif, and their contributions are only detectable *in vivo*.

5.3.3 A Unique DNA Sequence Is Recognized by the Nrf2-Small Maf Heterodimer

As an obligatory heterodimeric partner molecule for CNC proteins, small Maf plays a critical role in the determination of the unique DNA binding specificity of the heterodimer [103]. A functional ARE/EpRE site characteristically requires the conserved GC sequence [GCNNN(G/C)TCA(T/C)] (Fig. 5.2A), which distinguishes the ARE/EpRE from the binding sequences of other bZIP transcription factors, including AP-1 [2].

The MARE is unique in terms of length. The complete MARE is a 13-bp [TGCTGA^G/C_(G)TCAGCA] or 14-bp [TGCTGA^{GC}/C_(G)TCAGCA] palindromic sequence that is bound by Maf homodimers, whereas the dimers of other bZIP transcription factors, including Jun/Fos (AP-1), CREB, and C/EBP, recognize 7- or 8-bp palindromic sequences, for example, the TPA(12-*o*-tetradecanoylphorbol 13-acetate)-responsive element (TRE; TGA^G/C_(G)TCA) or the cyclic AMP-responsive element (CRE; TGA^{GC}/C_(G)TCA) [104, 105] (Fig. 5.2A). The ARE/EpRE is regarded as a composite sequence with a half-site of the typical MARE and a half-site of the typical TRE. Small Maf and Nrf2 recognize the former and the latter portions, respectively [103]. Recognition of the GC bases flanking the core TRE or CRE is a unique feature of the small Maf-DNA interaction, which critically distinguishes the ARE/EpRE from a TRE or CRE.

The structural basis of the small Maf-DNA interaction was clarified through the crystallization of the bZIP motif of MafG and the MARE-containing DNA duplex [106]. Two structural components are specific to small Maf proteins. One is the presence of the EHR on the N-terminal side of the basic region, and the other is the tyrosine residue (Tyr-64) in the basic region, whose corresponding residue is alanine in other bZIP transcription factors (Fig. 5.7). The overall structure of MafG homodimers and DNA is almost identical to that of

AP-1 (c-Jun and c-Fos heterodimer), and the two Maf-specific components are not directly involved in the recognition of the GC bases outside of the TRE core. Maf-specific structural elements contribute to the unique orientation of the side chains of the invariant amino acids in the basic region, Arg-57 and Asn-61, which play direct roles in establishing unique DNA recognition specificity.

	GCN4	PAALKRARNTTEAARRSRARKL
	ATF-4	KKLKKMEQNKTAATRYRQKKR
	c-Jun	KAERKRMNRNIAASKCRKRKL
	c-Fos	KRRIRREERNKMAAAKCRNRRR
CNC family	CNC	RDILRRRGKNKVAAQNCRKRKL
	NF-E2	RDILRRRGKNKVAAQNCRKRKL
	Nrf1	RDILRRRGKNKMAAQNCRKRKL
	Nrf2	RDILRRRGKNKVAAQNCRKRKL
	Nrf3	RDILRRRGKNKVAAQNCRKRKL
	Bach1	HDILRRRSKNRIAAQRCRKRKL
	Bach2	HDILRRRSKNRIAAQRCRKRKL
Maf Family	c-Maf	KQKRRTLKNRGYAQSCRFRV
	MafA	KQKRRTLKNRGYAQSCRFRV
	MafB	KQKRRTLKNRGYAQSCRYKRV
	Nrl	KQRRRTLKNRGYAQACRSKRL
	MafG	KQRRRTLKNRGYAASCRVKRV
	MafK	KQRRRTLKNRGYAASCRIRRV
	MafF	KQRRRTLKNRGYAASCRVKRV

Fig. 5.7 Alignment of the basic domains of various bZIP transcription factors. The tyrosine residue is unique to Maf family proteins and corresponds to the alanine residue of the other bZIP proteins.

Comprehensive measurement of the binding affinities to MARE and related sequences was performed using the MafG homodimer and the Nrf2-MafG heterodimer [103, 107]. The comparison between the two results revealed that the MARE-related sequences can be categorized into three groups: homodimer-oriented MAREs, heterodimer-oriented MAREs, and ambivalent MAREs (Fig. 5.8). Homodimer MAREs are considered to be the exclusive targets of Maf proteins, while heterodimer MAREs are considered to be the exclusive targets of CNC-small Maf. The ambivalent MAREs should be bound competitively by both homodimers and heterodimers (Fig. 5.8). However, the *in vivo* significance of MARE-related sequence categorization into three classes remains to be evaluated.

Interestingly, swapping the unique tyrosine residue of MafG and the corresponding alanine residue of Nrf2 resulted in the simultaneous swapping of DNA recognition specificity [108]. The Nrf2 A502Y-MafG heterodimer preferentially recognizes homodimer-oriented MAREs as well as ambivalent MAREs (Fig. 5.8). Comparison of the target genes of the Nrf2 A502Y-MafG heterodimer with those of the wild-type Nrf2-MafG heterodimer demonstrates the significance of the distinct DNA recognition specificity of the Maf homodimer and the CNC-Maf heterodimer.

5.3.4 Regulation of Small Maf Expression

Genes encoding the small Maf proteins, MafF, MafG, and MafK, are expressed in a wide range of tissues [90, 96, 109, 110]. Dissection of the regulatory region of the *MafK* gene led to a new concept in which ubiquitous

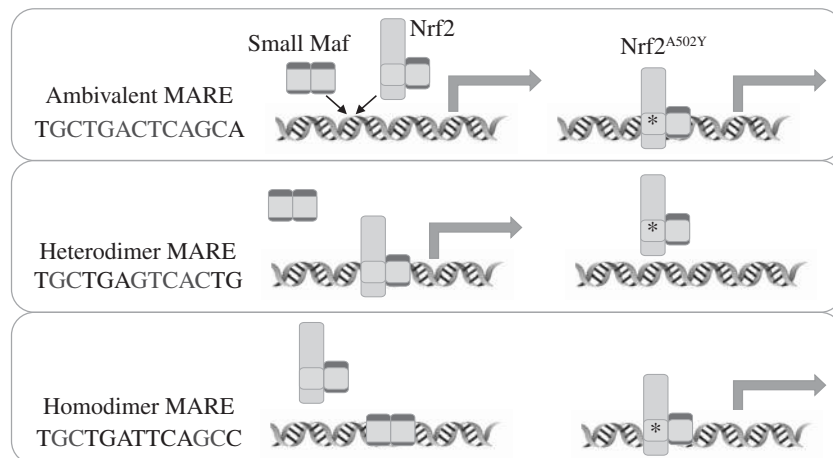


Fig. 5.8 Categorization of MARE-related sequences. Ambivalent MAREs are bound by both Nrf2-small Maf heterodimers and Maf homodimers. Heterodimer MAREs are preferentially bound by Nrf2-small Maf heterodimers, and homodimer MAREs are bound by Maf homodimers. Substitution of the alanine residue of the Nrf2 basic region with a tyrosine residue switches the DNA recognition specificity from the CNC type to the Maf type and results in the preferential binding of Nrf2 A502Y-small Maf heterodimers to homodimer MAREs and not to heterodimer MAREs. (See color insert.)

expression is achieved by the coordinate function of multiple tissue-specific enhancers rather than the activity of a single general enhancer [109, 111, 112]. Interestingly, many stimuli that induce Nrf2 activation enhance transcription of the *MafG* gene, including hydrogen peroxide, β -naphthoflavone, cadmium, zinc, and arsenite [113–115]. Consistently, the *MafG* gene possesses an ARE/EpRE in its promoter and is directly activated by Nrf2 [116], which constitutes a positive feedback loop. An increase in small Maf proteins must accompany Nrf2 stabilization for efficient use of the available Nrf2. The *MafG* gene is also induced by hypercapnic stimulation in the medulla oblongata by inhalation of 7% CO₂, where the central baroreceptive neurons are distributed [117]. Induction of the *MafG* gene is observed upon activation of the baroreceptors by the repressor agent phenylephrine [118]. The precise mechanism of *MafG* induction via these challenges in neural cells is still unknown.

5.4 DYSFUNCTION OF NRF2 IN PATHOLOGICAL CONDITIONS

Nrf2 dysfunction has been implicated in many pathological conditions. Functional impairment of Nrf2 is related to the susceptibility to exogenous chemical and physical insults and to the proinflammatory predisposition. Nrf2 inducers are beneficial for the treatment of these conditions. Naturally occurring electrophiles contained in vegetables and other synthetic drugs have been shown to be effective Nrf2 inducers. By contrast, aberrant hyperfunction of Nrf2 has been found in various human cancers. Nrf2 inhibitors, now under development, are expected to make promising anticancer drugs that increase the efficacy of chemotherapy and radiotherapy and inhibit the proliferation of cancer cells.

5.4.1 Dual Functions for Nrf2 in Cancer Pathology

Because Nrf2 is a key regulator of the inducible expression of detoxifying enzymes, chemical challenge produces more severe damage to *Nrf2*-null mice than to wild-type mice. One of the most obvious adverse effects in *Nrf2*-null mice is susceptibility to chemical carcinogenesis [23, 65, 119–122]. In the absence of Nrf2, elimination of highly reactive metabolites of chemicals is delayed, resulting in the increased formation of DNA adducts and the subsequent accumulation of DNA mutations. Nrf2 plays a protective role in carcinogenesis.

In contrast, Nrf2 expression in cancer cells contributes to malignant phenotypes during cancer progression and leads to a poor prognosis [70]. Somatic mutations in the *KEAP1* gene or the *NRF2* gene are found in a substantial portion of human cancers, including lung cancer,

gallbladder cancer, head and neck cancer, and breast cancer [68–70, 73, 123–125] (Fig. 5.5). Reduced expression of *KEAP1* due to DNA methylation of the promoter region of the gene has been reported in lung cancer and prostate cancer [75, 76]. These genetic and epigenetic alterations cause constitutive stabilization of Nrf2 and elevated expression of cytoprotective genes in cancer cells. Constitutive stabilization of Nrf2 and sustained expression of its target genes confer resistance to anticancer drugs, such as cisplatin, carboplatin, etoposide, and 5-fluorouracil [68–70] and irradiation [126].

A recent study demonstrated the protective role of Nrf2 against cancer metastasis [127]. Nrf2 activity in cells of hematopoietic origin, which are thought to be myeloid-derived suppressor cells, is critical to the inhibition of lung metastasis of cancer cells. Thus Nrf2 is a double-edged sword in cancer pathology; Nrf2 activation in the host is beneficial, while Nrf2 activation in the cancer is detrimental.

5.4.2 The Contribution of Nrf2 to Inflammatory Processes

The Keap1-Nrf2 system responds not only to xenobiotic electrophiles but also to endogenous electrophiles, including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) [128], 8-nitroguanosine 3',5-cyclic monophosphate (8-nitro-cGMP) [41, 61], 4-hydroxynonenal (4-HNE) [129], and the oxidation products of omega-3 fatty acids [130–132]. 15d-PGJ₂ is generated during inflammatory responses in which cyclooxygenase-2 (COX-2) plays a critical role. 8-Nitro-cGMP is generated in the process of nitric oxide signaling, and 4-HNE is one of the products of lipid peroxidation caused by ROS. Derivatives of omega-3 fatty acids, which have received much attention because of their anti-inflammatory nature, are produced in the resolution phase of inflammation. Thus Nrf2 should be activated in processes in which these signaling pathways are operative.

Indeed, Nrf2 plays a critical role in the resolution phase of inflammation, when COX-2 activity is increased [128]. Carrageenan-induced pleurisy is aggravated and prolonged in *Nrf2*-null mice. Pleural macrophages express high levels of COX-2 in the resolution phase of acute inflammation, which promotes the synthesis of 15d-PGJ₂. Subsequent activation of Nrf2 facilitates the remission of cell infiltration [128, 133]. Nrf2 deficiency confers a predisposition toward inflammation, resulting in the exacerbation of emphysematous changes following insults from cigarette smoke or elastase [19, 133–135] and of fibrotic changes following bleomycin exposure [136]. *Nrf2*-null mice also display susceptibility to ConA-induced acute inflammatory liver injury [137]. In many cases, ablation of *Nrf2* in

mice results in upregulation of proinflammatory cytokines, implying an excessive inflammatory reaction in *Nrf2*-null mice (Table 5.1).

5.4.3 Nrf2 in Cell Proliferation and Tissue Regeneration

Microarray experiments have revealed that Nrf2 regulates not only detoxifying enzymes and antioxidant proteins but also several factors promoting cell proliferation [138–140]. Constitutive activation of Nrf2 promotes cell proliferation [68, 69], while disruption of the *Nrf2* gene compromises cell proliferation [139]. *Nrf2*-null cells display impaired cell cycle progression accompanied by a reduction in the phosphorylation of Akt and the protein abundance of ERK2 and Stat3 [141]. Importantly, supplementation of glutathione rescued the impaired proliferation, the compromised cell cycle progression, and the reduced kinase activities of *Nrf2*-null cells [139, 141]. Nrf2 sustains a high rate of cell proliferation by replenishing glutathione efficiently.

A new role for Nrf2 has been implicated in the process of liver regeneration [142, 143]. *Nrf2*-null mice showed delayed initiation of liver regeneration and impaired Akt phosphorylation following transient activation of insulin signaling after hepatectomy [142]. Notch1 was found to be a Nrf2 target gene responsible for liver regeneration [143]. Hepatocyte-specific expression of the intracellular domain of Notch1 rescued the impaired liver regeneration in *Nrf2*-null mice.

5.5 CONCLUSION AND FUTURE PERSPECTIVES

Because Nrf2 confers resistance against electrophilic and oxidative stress, Nrf2 activation by chemical compounds and natural products is a promising strategy for disease prevention and therapy. Synthesized compounds and natural products that activate Nrf2 have been tested for cancer chemoprevention. Oltipraz, dithiolethiones, triterpenoids, curcumin, and sulforaphane have been reported to be effective Nrf2 inducers [144–147]. The pharmacological effects of these chemicals are mediated by Nrf2, and they are ineffective in the absence of Nrf2 [65, 119, 146, 148]. The most potent Nrf2 inducer that has been identified to date is CDDO-Im (1-[2-cyano-3, 12-dioxoooleana-1, 9(11)-dien-28-oyl]imidazole), a synthetic triterpenoid. The effective dose of CDDO-Im for the prevention of carcinogen-induced hepatic and lung tumors is much lower than that of sulforaphane and oltipraz [148, 149]. In addition, administration of CDDO-Im attenuates cisplatin-induced renal damage [150], retinal injuries [151], and hyperoxia- and smoke-induced lung injuries [152, 153].

In contrast to Nrf2 inducers, few specific inhibitors of Nrf2, which are expected to be beneficial for anticancer therapy, have been reported. One of the most difficult but important issues in the development of Nrf2 inhibitors is how to achieve specificity. Recently, a compound purified from plant extracts, brusatol, was found to enhance the degradation of Nrf2 and inhibit Nrf2-mediated stress response and tumor growth [154]. The development of Nrf2 inhibitors based on the molecular mechanism of the Keap1-Nrf2 system is one of the most critical and challenging tasks in the development of effective therapies for cancer.

Another important issue that needs to be pursued is the cross talk between the redox response of Keap1 and other signaling pathways in the activation of Nrf2-mediated transcription. Activation of Nrf2 appears to be regulated by phosphorylation-signaling pathways, including mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), PKC, and casein kinase 2 (CK2) [155]. Ser40 and Tyr568 have been identified as phosphorylation sites of Nrf2 by PKC and the tyrosine kinase Fyn, respectively [83, 84, 87, 129]. p21 and p62 were identified as nonelectrophilic, endogenous inducers of Nrf2 [77, 80]. The former is a target gene of p53-mediated cell cycle arrest, and the latter is a polyubiquitin-binding protein targeting various substrates for autophagy. Elucidation of the physiological and pathological significance of the interaction between these pathways and the Keap1-Nrf2 system is an important future goal for the attainment of an overall understanding of mammalian stress response signaling.

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CALORIC RESTRICTION AND OXIDATIVE STRESS

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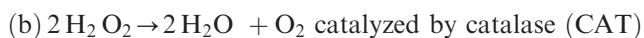
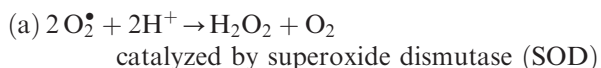
6.1 INTRODUCTION

Living organisms create reactive oxygen species (ROS) like superoxide anion, hydroxyl radical, hydrogen peroxide, and nitrogen oxide radical. These short-lived intermediates are deleterious for cells and tissues, and their removal by a scavenger system involving either endogenous or exogenous substances is therefore important. Both processes, ROS generation and inhibition, are well balanced in healthy organisms, but either increased ROS production or insufficient defensive mechanisms create impairment in cell signaling.

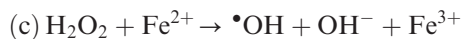
ROS may contribute to development of different pathological disorders such as inflammation, diabetes, atherosclerosis, and tumors because ROS play major role in their pathogenesis. In addition, the most important theory concerning pathogenesis of aging is based on oxidative stress as well [1–3]. This hypothesis was formulated on the observation that (a) overexpression of antioxidative enzymes slows down the age-related oxidative damage and extends life span in experimental animals, (b) variations in longevity among species inversely correlate with mitochondrial generation of ROS, and (c) restricted caloric intake decreases levels of oxidative stress, retards the age-related changes, and extends life span in mammals [1]. Overnutrition or caloric restriction significantly affects not only the level of oxidative stress but also cell functions and the life span of living organisms. Elucidation of nutrition-related mechanisms of oxidative stress changes and their cell/tissue consequences may support lifestyle recommendations.

6.2 OXIDATIVE STRESS—BASIC CHARACTERISTICS

In physiological conditions each cell produces short-living intermediates known as ROS. Different intermediates or molecules including both radicals like superoxide, hydroxyl, or peroxy radicals and reactive molecules like hydrogen peroxide have been identified. They are created from oxygen-containing molecules and free electrons. Superoxide radical may be formed by one-electron reduction of oxygen, whereas two-electron reduction produces hydrogen peroxide. The intermediates are quenched by antioxidant scavenger enzymes with subsequent formation of neutral molecules (e.g., water):



Hydrogen peroxide has the potential risk to produce hydroxyl radical formed in the presence of transition metals like copper or iron.



ROS often initiate chain reaction when electrons are passing to other molecules (saccharides, lipids, proteins, or DNA), by creating their oxidative products (e.g., lipoperoxides). This may have deleterious effects on

their function, such as gene mutations or inflammation initiating tissue pathology and/or different diseases (diabetes, atherosclerosis, tumors, etc.).

The removal of ROS is therefore important to maintain physiological reactions within the body and to prevent development of pathological processes. The scavenger system is the important counterpart possessing sufficient capacity under normal conditions to quench the ROS intermediates or molecules. Both enzymatic and nonenzymatic systems are present in the body. In addition to enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), or glutathione reductase (GR), there are antioxidant molecules like tocopherol, ascorbic acid, uric acid, bilirubin, etc. possessing antioxidative properties. These are contained in body fluids, and a specific role in oxidative stress has been elucidated in some of them.

ROS formation may be accelerated by internal and external factors. The former include abnormal processes represented by inflammation when released cytokines activate some regulatory molecules [e.g., nuclear factor kappa B (NF- κ B)] with consequently created ROS. However, in the most pathological processes the ROS are both a consequence of the cascade events and a promoter of the pathology. Pathogenesis of the disease thus combines different mechanisms including ROS formation that cannot be separated from other reactions because the whole process is highly complex. The power of protective mechanisms represented by scavenger enzymes may significantly determine the level of

oxidative stress. On the other hand, external factors like food may influence oxidative stress either by supply of compounds accelerating ROS formation [by advanced glycated end products (AGEs)] or of protective molecules (antioxidant vitamins, polyphenols like resveratrol).

The final status of oxidative stress in the body is rather complicated and dependent on the interrelationship of all endogenous and exogenous influences.

6.3 MITOCHONDRIA—THE MAIN CELL REACTIVE OXYGEN SPECIES GENERATOR

One of the main source of ROS is the respiratory chain in mitochondria in which the electron flux passing complexes I, II, III, and IV is associated with formation of superoxide radical (Fig. 6.1) [4–6]. Under physiological conditions the substrates metabolized in the tricarboxylic acid cycle generate electron donors like NADH and FADH₂. The former gives electrons to complex I, the latter to complex II. Then the electron flux continues to coenzyme Q and further to complex III, cytochrome *c*, complex IV, and finally to molecular oxygen with generation of water.

In parallel, the protons are pumped from the mitochondrial matrix into the mitochondrial intermembrane space, generating an electrochemical gradient. This membrane potential with protons outside the inner membrane is a driving force for phosphorylation of ADP to ATP by ATP synthase (complex V), which pumps the protons

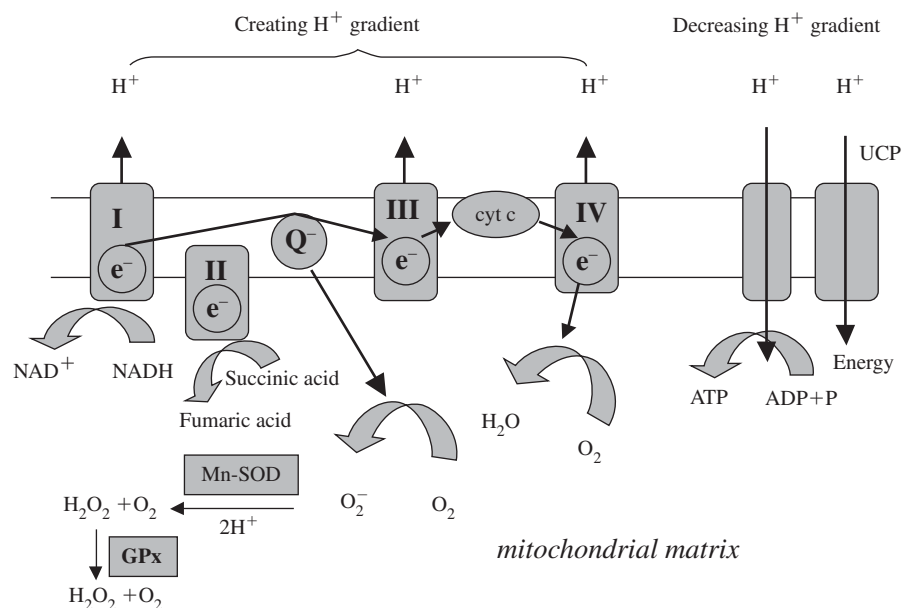


Fig. 6.1 Superoxide radical generation in the respiratory chain of mitochondria: complexes I, II, III, and IV. UCP, uncoupling protein; Mn-SOD, manganese superoxide dismutase; GPx, glutathione peroxidase.

back to the mitochondrial matrix and reduces the membrane potential. Complex V is a rate-limiting step in the generation of ATP in mitochondrial respiration. In addition, ATP generation may be attenuated by proton leak, which shuttles the protons from the intermembrane space to the mitochondrial matrix and thus reduces the number of protons flowing through the ATP synthase. A part of this proton leak is operated by uncoupling proteins (UCPs) regulating the flux of protons through the ATP synthase. The greater level of ATP may inhibit mitochondrial respiration, and therefore uncoupling the proton flux by UCPs is an adaptive mechanism to avoid inhibition of the respiration.

The main source of free radicals is the mitochondrial respiratory chain, where oxygen radical generation has been attributed to complexes I and III. The importance of complex I for ROS production in short-lived compared with long-lived species has been repeatedly documented [7, 8]. The ROS generator was localized within the FeS clusters placed in the hydrophilic matrix domain of the complex I [5, 8], whereas ROS generation within complex III is directed to the cytosolic site [9]. Different localization may explain the finding that mitochondrial DNA (mtDNA) oxidative damage is more common when the ROS are generated by complex I.

The previous hypothesis that ROS production depends on oxygen consumption has not been confirmed. There is clear evidence that mitochondrial membrane potential is a major factor that determines ROS production [10]. Membrane potential may be lowered in the presence of mitochondrial uncouplers or inhibitors. An increased mitochondrial UCP (UCP3) content was accompanied by a lower rate of ROS production [11]. With the use of the UCP2 inhibitor guanosine diphosphate (GDP) an increase in membrane potential and H_2O_2 production has been observed as a consequence of diminished proton leak [12, 13]. Similarly in plant mitochondria, the greater activity of mitochondrial UCPs decreases membrane potential and inhibits formation of ROS at the level of coenzyme Q [14].

The relationship between ROS and UCPs regulating ROS production has been observed [15]. Greater production of superoxide was capable of activating the proton conductance of UCPs and then diminishing superoxide formation as self-mediated feedback [16]. Reactive alkenals created by lipid peroxides have been proposed as the causative factor for UCP activation. Interactions between ROS and UCPs demonstrate a possible mechanism by which free radical concentrations inside the mitochondria may regulate their own production. Any impairment of UCPs decreasing their effectiveness in diminishing membrane potential by increasing proton leak may accelerate superoxide production in mitochondrial matrix [13].

Mitochondrial dysfunction is an important component of aging, type 2 diabetes, neurodegenerative disorders like Alzheimer and Parkinson diseases, and cancers [17, 18]. Accelerated oxidative stress has been considered as the main pathogenic process associated with mitochondrial dysfunction [3]. Superoxide and hydrogen peroxide are two main ROS produced in mitochondria. The majority of mitochondrial superoxide (70–80%) is released to the mitochondrial matrix, whereas the remaining 20–30% is released into the intermembrane space [3]. Intramitochondrial manganese superoxide dismutase (MnSOD) catalyzes the superoxide transformation into hydrogen peroxide inside the mitochondrial matrix. Hydrogen peroxide production is modulated by the mitochondrial metabolic state and by the intramitochondrial concentration of nitric oxide (NO). The rates of hydrogen peroxide production are affected by ion movements through the inner mitochondrial membrane. Its removal is catalyzed in mitochondrial matrix by GPx transforming its molecule into water and oxygen.

NO is produced by NO donors or by nitric oxide synthase (NOS) localized within the mitochondria (mtNOS), although there is still some controversy concerning its existence [3]. The enzymatic reaction requires arginine, NADPH_2 , and O_2 as substrates and produces citrulline, NO, and H_2O (Fig. 6.2). NO inhibits complex III electron transfer and increases superoxide and hydrogen peroxide production. NO is transformed to peroxynitrite, which is a strong oxidant and inhibitor of both complexes I and III. Peroxynitrite remains in the intramitochondrial space and leads to mitochondrial dysfunction and apoptosis. It is the source for nitration of

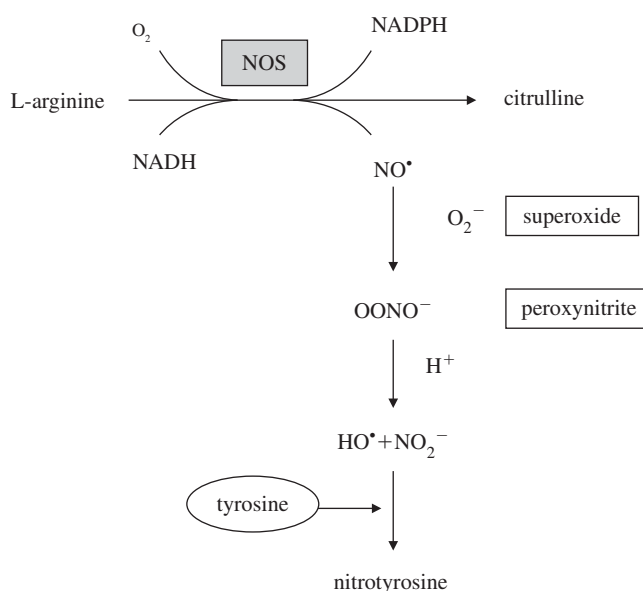


Fig. 6.2 Nitric oxide and nitrotyrosine generation. NOS, nitric oxide synthase.

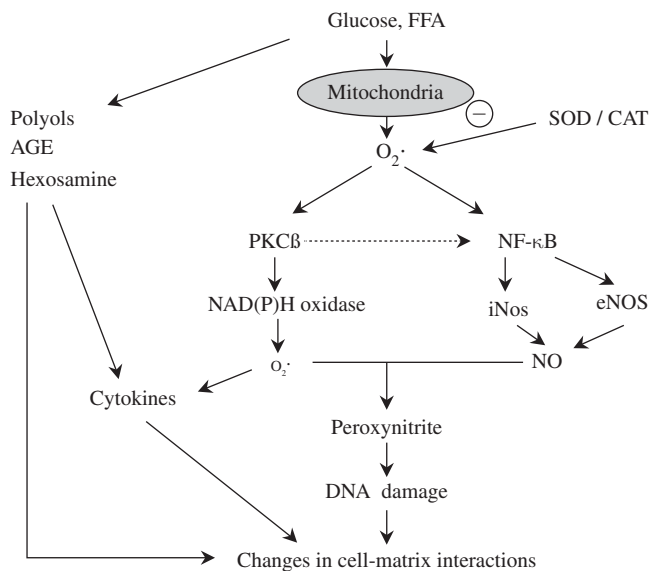


Fig. 6.3 Generation of superoxide by endogenous glucose and free fatty acids (FFA) and relationship to reactive nitrogen species. AGE, advanced glycation end products; SOD, superoxide dismutase; CAT, catalase; PKC β , protein kinase C β ; NF- κ B, nuclear factor κ B; NOS, nitric oxide synthase.

tyrosine residues in proteins and peptides. Nitrotyrosine may be detected as the resulting product.

Oxidative damage is induced by ROS produced primarily as a by-product of mitochondrial oxidative phosphorylation, which is responsible for 85–90% of cellular oxygen consumption [19]. Mitochondrial ROS can cause damage to mtDNA, proteins, and membrane lipids, and thus it contributes to functional and morphological changes observed in pathological states (Fig. 6.3). This process has a self-perpetuating cycle character because increased ROS production leads to incremental damage and further ROS generation [20].

In addition, localization of the ROS production in mitochondria explains why mtDNA is damaged more than nuclear DNA [21]. The rate of oxygen radical attack on mtDNA contributes to differences between long-lived and short-lived animals [22]. A higher rate of oxidative attack in mtDNA was found in short-lived than in long-lived animals, and a similar situation was true with the repair. Oxidative damage of mtDNA may be measured by 8-hydroxy-2'-deoxyguanosine (8-OH-dG), correlating inversely with longevity in birds and mammals [21]. This relationship was not found in the case of nuclear DNA. Mutations in mtDNA caused by ROS are deleterious for cells. The alterations include depressed respiration, enhanced radical formation, increased susceptibility to oxidative stress-triggered apoptosis, accumulation of mutant mitochondria inside the cells, and ROS secretion by mutated cells. These changes observed in short-lived animals prove the role of mtDNA mutations in

acceleration of aging [23]. Increased aging rate due to frequent mtDNA mutations was directly demonstrated in mice [24]. Impaired lysosomal degradation of oxidatively damaged mitochondria can also contribute to the aging process [25]. A low rate of mitochondrial ROS production accompanied by low levels of oxidatively damaged mtDNA may therefore delay the aging process. This was demonstrated in different organs including brain, heart, and liver.

Regulation of ROS production is therefore the most important role of mitochondria, thus influencing the aging process and preventing different ROS-dependent disorders [26]. Dietary manipulations involving caloric or methionine restrictions or supplementation with resveratrol may significantly decrease deleterious effects of ROS/reactive nitrogen species (RNS) by improving their homeostasis.

6.4 REACTIVE OXYGEN SPECIES GENERATED BY FOODS

The pandemic of obesity in developed countries has resulted in extensive research of its risk factors and their consequences at the molecular level. A modern lifestyle involving low physical activity as well as overeating characterized by high calorie intake are leading factors in overweight and obesity development. Changes in the ROS production and antioxidant mechanisms have been observed in obese persons, and the possible role of both exogenous factors, physical inactivity and overeating, has been intensively studied. Repeated results obtained in both animal and human studies have demonstrated that physical exercise and decreased food intake confer favorable effects on subjects. The effect of ingested calories on ROS production has been confirmed in experimental and clinical studies (Fig. 6.4).

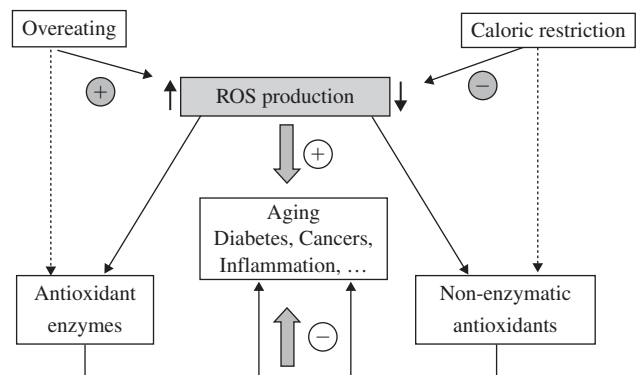


Fig. 6.4 Effect of eating habits on ROS production and its relationship to ROS-mediated diseases.

Higher caloric intake generates more ROS by two mechanisms. First, food that increases body fat, mainly visceral, associated with insulin resistance, subclinical inflammation, and activation of cytokines from adipose tissue causes conditions of greater ROS generation. However, the adipose tissue itself is not the only source of ROS. There are several pathways participating at this process. Adipose tissue resistant to insulin releases more free fatty acids (FFAs) into the bloodstream, which transports them into various organs. The liver, pancreas, heart, vessel walls, and muscles are exposed to the overload of fatty acids, and ectopic fat is then created. More substrates coming into the cells accelerate the Krebs cycle and consequently the supply of NADH and FADH₂ into the respiratory chain in mitochondria. FFAs not only cause insulin resistance in the above organs but create more ROS either directly or by activation of the protein kinase C (PKC) rare isoforms (Fig. 6.5) [27]. ROS are closely related to subclinical inflammation by the bridge of activated nuclear factor NF- κ B.

Second, food composition and type of preparation may significantly modify the ROS content within the body [28]. Exogenous AGEs produced in greater amounts by broiling and frying of food increase the AGE plasma level and act as contributors of ROS after ingestion of the meal [29] (Fig. 6.6). The AGEs are

recognized by specific membrane receptors on macrophages or endothelial cells [receptors for AGEs (RAGEs)], which are then activated to produce cytokines and inflammatory proteins [30]. Alternatively, the AGEs are bound in plasma to soluble receptors (sRAGEs) containing extracellular domain released by

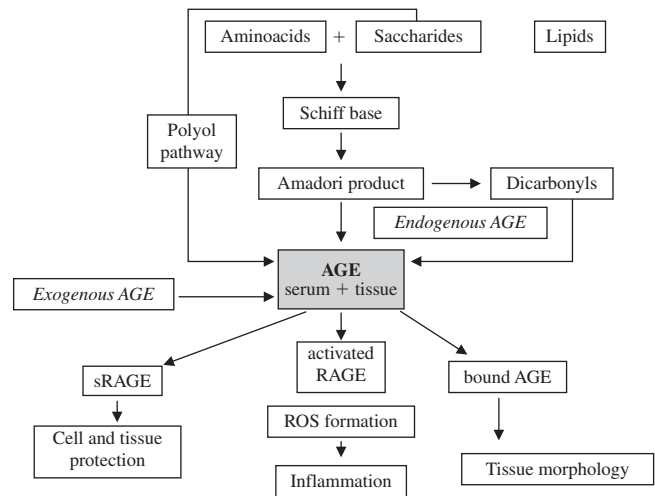


Fig. 6.6 Role of endogenous and exogenous advanced glycation end products (AGE) in ROS formation due to RAGE activation.

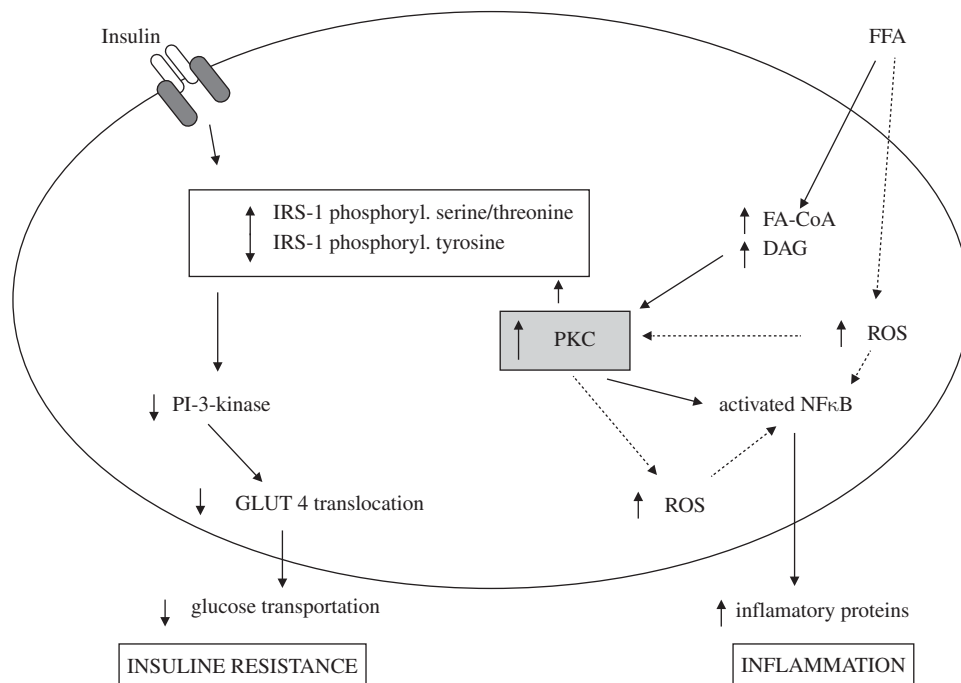


Fig. 6.5 Central role of protein kinase C (PKC) isoforms in the effect of free fatty acids (FFA) on muscle cell ROS production and insulin resistance development. IRS, insulin receptor substrate; PI-3-kinase, phosphatidylinositol 3-kinase; FA-CoA, fatty acid coenzyme A; DAG, diacylglycerol.

metalloproteinases from membrane-bound RAGEs [31]. These complexes circulating in plasma preserve the AGE target cells from activation and prevent both ROS formation and inflammation. Lower sRAGE capacity in individuals with caloric overload may activate target cells to ROS production. Foods containing fats show the highest amount of AGE content [28], and thus they contribute to ROS formation. Acute vascular dysfunction due to AGEs in the food was described in the postprandial state [32]. Excessive AGE consumption represents an independent factor for inappropriate oxidant stress responses, which may promote the premature expression of complex diseases associated with adult life, such as diabetes and cardiovascular disease. ROS may further modify saccharides and lipids by forming glycoxidation products or lipid peroxides that contribute to pathogenesis of atherosclerosis and aging. This process has an autocatalytic character that potentiates the deleterious effect of high saccharide or fat consumption (Fig. 6.7) [33]. Dicarbonyl glycation damage to the mitochondrial proteome may be a preceding event for mitochondrial failure leading to oxidative stress [34].

Some molecular mechanisms associating ROS formation and advanced aging have been recognized. Superoxide and other ROS inside the cell are activators of poly(ADP-ribose) polymerase 1 (PARP1), which is a chromatin-associated nuclear protein acting as a molecular stress sensor [35]. PARP1 enzyme activity is then strongly advanced, and poly(ADP-ribose) may be created. This process consumes NAD^+ , which is lacking in a large number of $\text{NAD}(+)$ -dependent enzymes, among which sirtuins are more prominent [36]. In addition, PARP1 acts as transcriptional cofactor for NF- κ B-dependent gene expression closely associated with inflammation mentioned above.

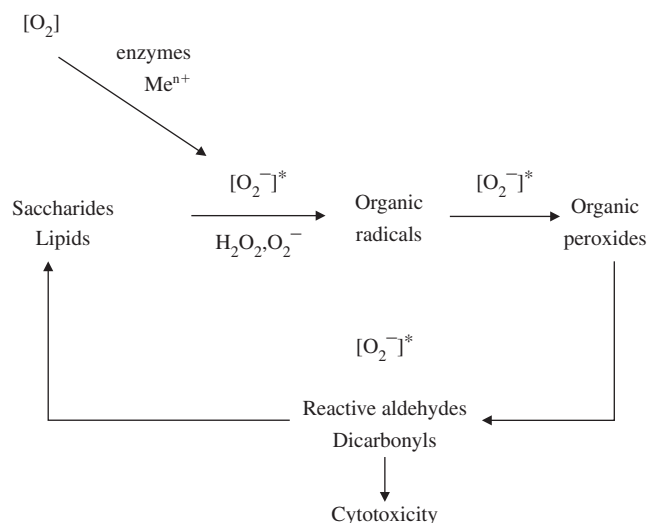


Fig. 6.7 Autocatalytic cycle of oxidative stress.

Caloric overload is therefore a great generator of ROS formation with consequent modification of saccharides, lipids, and proteins. Dietary restriction is the most powerful modulator of the aging process in diverse groups of organisms. Its multifaceted effects are achieved by potentiating the immune responses, lowering oxidative stress, acting as a neuroprotector, and attenuating inflammatory processes [3, 37]. Dietary restriction therefore has robust effects on delaying mortality, increasing life span, and attenuating chronic diseases of old age. It may be important to prevent diseases in older age and to promote healthy aging in humans.

6.5 OXIDATIVE STRESS MODULATED BY CALORIC RESTRICTION AND OTHER FACTORS

A large amount of clinical and experimental data obtained during the last decade demonstrates that overfeeding accelerates ROS production and consequently increases different vascular complications in obese subjects. In addition, it promotes aging rate and lowers longevity. On the other hand, restriction of energy intake was accepted as the main therapeutic principle offering better prognosis to obese persons. Animals under caloric restriction without malnutrition maintain most physiological functions in a youthful state at more advanced ages. Caloric restriction retards age-related diseases such as diabetes, cardiomyopathy, nephropathy, hypertension-related diseases, and tumors [38]. The beneficial effects can be observed when caloric restriction is started both at a young age and in middle age or later [39]. Ongoing studies demonstrate that caloric restriction may reduce the aging rate in rodents [40] as well as in primates [41]. All these promising results motivate investigation to elucidate molecular mechanisms participating in changes of tissues, organs, or organisms as well.

6.5.1 Role of Sirtuins

Genetic and molecular studies demonstrate that low caloric intake is a regulated process with the silent information regulator 2 (Sir 2) gene playing an important role. This is a member of the sirtuin family that mediate different physiological effects and thus influence aging, metabolic diseases, or tumorigenesis [36]. Seven sirtuins (SIRT1–SIRT7) have been described in mammals, belonging to the protein-modifying enzymes known as NAD-dependent histone deacetylases [42–44] that catalyze hydrolysis of acetyllysine [45]. This reaction consumes NAD and releases nicotinamide, *O*-acetyl ADP ribose, and deacetylated substrate. Three sirtuins have been localized in the nucleus, three in

mitochondria, and only one in cytoplasm [46]. Mammalian SIRT1 has several effects in glucose homeostasis [47], insulin secretion [48, 49], and lipid mobilization [50]. A much wider role of sirtuins is suggested in physiological processes including lifespan regulation and cellular response to stress [51].

Reduced energy intake upregulates SIRT1 in muscle, fat, and liver [51]. SIRT1 regulates hepatic glucose metabolism by stimulating gluconeogenesis, which is mediated by peroxisome proliferator-activated receptor (PPAR) coactivator (PGC)-1 α at the level of gene transcription [47]. SIRT1 stimulation of gluconeogenesis opposes the insulin effect in the liver promoting gluconeogenesis suppression. Insulin-resistant states are associated with decreased SIRT1 expression and increased expression of UCP2. SIRT1 inhibition may induce adipogenesis by activating PPAR γ , whereas glucose signaling and consequently insulin secretion from β -cells in pancreas are reduced. On the other hand, activation of SIRT1 decreases adipocyte formation during osteoblast differentiation from mesenchymal stem cells [52].

Because of the significant role of sirtuins, inhibitors and activators of their expression have been studied. Sirtuin inhibitors may increase p53 activity that stops the formation of tumors and induces apoptosis [53]. Inhibitor will be reliable in the treatment of neurodegenerative Parkinson disease. Sirtuin activators increase life span and cell survival, promote fat mobilization, and increase mitochondrial size and number [54]. Sirtuins link nutrient availability and energy metabolism. Sirtuins, like “molecular sensors,” mediate the effects of caloric restriction on the aging process. The most potent activator among natural compounds is resveratrol, a polyphenol antioxidant. Significant reduction of cellular hydrogen peroxide, upregulated MnSOD expression, and increased cellular glutathione content have been observed after resveratrol administration [55]. It is proposed that resveratrol upregulates antioxidant defense mechanisms and attenuates mitochondrial ROS production via sirtuin activation. This effect makes resveratrol a potent neuroprotectant and antiapoptotic agent [56]. Resveratrol acts as a phytoestrogen mimicking the activity of 17 β -estradiol and interacting with estrogen receptors α and β (Fig. 6.8; see also section 6.5). Its administration caused significant increase of MnSOD gene expression and augmented its activity several times in human cells [57].

Association of sirtuins with antioxidative defense is proposed by different experimental and human studies [58], although the molecular mechanisms have not been elucidated yet. The relationship between sirtuin activation and SOD transcription has been described [59]. Sirtuins are proposed as mediators of antioxidative mechanisms promoting a decreased level of oxidative stress. A strong association of sirtuin (SIRT1) alterations

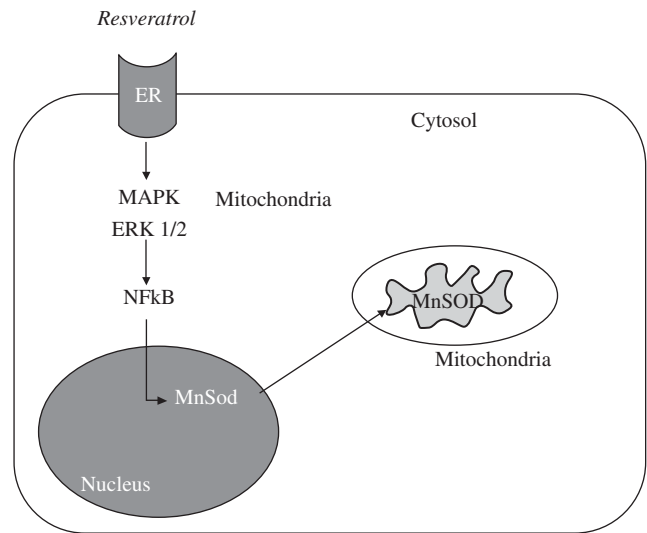


Fig. 6.8 Stimulation of manganese superoxide dismutase (MnSOD) synthesis by resveratrol via estrogen receptor. MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal regulated kinases 1/2; NF- κ B, nuclear factor κ B.

with changes in AMP-activated protein kinase (AMPK) has been proposed in an experimental study [60]. SIRT3 has been suggested as an essential player in enhancing the mitochondrial glutathione antioxidant defense system during caloric restriction [61]. SIRT3-dependent mitochondrial adaptation may be a central mechanism retarding aging in mammals [61]. Besides its molecular effects, SIRT1 is a key regulator of vascular endothelial homeostasis controlling angiogenesis, vascular tone, and endothelial dysfunction [62]. A possible link between vascular effects and ROS generation has been suggested.

Sirtuins are potential mediators of caloric restriction and have a promising role as pharmacological targets to delay aging and age-related diseases [63].

6.5.2 Caloric Restriction

Caloric restriction has a protective effect on oxidative damage due to a reduced rate of mitochondrial free radical generation as demonstrated in the rat liver [64], heart [65], skeletal muscle [66], and brain [67]. In many experimental studies 40% reduction in caloric intake was used to demonstrate beneficial effects on reduced ROS production observed already after 7 weeks [68]. Decreased mitochondrial ROS production at complex I of the respiratory chain was found in different studies. However, more detailed studies showed that decreased mitochondrial ROS production is a function of dietary composition. While protein restriction caused a significant decrease in the ROS generation and oxidative damage in mtDNA, no such effects were found after a

carbohydrate- or fat-restricted diet [69–72]. Lowered dietary protein intake and not the calories themselves was responsible for the decreased mitochondrial ROS generation [73]. Special attention was given to methionine restriction, which seems to be responsible for the decreased ROS production observed in a calorie-restricted diet [69, 73, 74]. Methionine supplementation increased mitochondrial ROS generation and percent free radical leak in rat liver mitochondria but not in the heart [75]. Lowering of methionine levels controls oxidative stress in mitochondria by two mechanisms: decreasing the sensitivity of proteins to oxidative damage and lowering the rate of ROS generation [69]. Forty percent calorie reduction may be too large for some persons, and therefore the effects of less restricted diets containing 8.5% and 25% restrictions have been examined. Neither 8.5% nor 25% caloric restriction changed the rates of mitochondrial ROS production or oxygen consumption in the rat liver mitochondria [76]. Opposing effects of a 25% calorie-restricted diet on respiratory chain complex I and III activities have been observed. Decrease in the complex I activity tended to decrease mitochondrial ROS production, whereas increase in the complex III tended to increase ROS generation. It seems that more than 25% caloric reduction is needed to lower the mitochondrial oxygen radical generation due to significantly reduced expression of complex I.

Caloric restriction reduces mitochondrial ROS production and promotes mitochondrial renewal via mitochondrial biogenesis and autophagy [77]. On the other hand, an experimental study in mice showed different results [78]. Caloric restriction increased lipid peroxidation, inflammation, and apoptosis, while decreased mitochondrial bioenergetic efficiency, protein oxidation, and stress response were found. The substantial upregulation of antioxidant enzymes and UCP3 could be a protective response to the heightened oxidative damage in mice. This study illustrates for the first time the detrimental effect of caloric restriction in mice, but no similar data are available in humans. In another study, Santos et al. [79] demonstrated a significant increase of hydrogen peroxide in Goto-Kakizaki and Wistar rats after food deprivation, with different responses in antioxidant enzymes GPx and Gred, which may contribute to oxidative imbalance in rat brain and thus to degeneration and death.

6.5.3 Fasting

Caloric restriction without malnutrition decreases ROS production and brings beneficial effects to subjects concerning life expectancy and delayed onset of several diseases. Although very short fasting has some benefits, prolonged fasting over 24 h may be harmful [80]. Severe

food deprivation increases oxidative stress by accelerating mitochondrial free radical generation and by increasing sensitivity of hepatic membranes to oxidative damage (lipid peroxidation). The oxidative changes are induced either by reactive carbonyl compounds or through amino acid oxidation [80]. Starvation induces superoxide anion release from the hepatocytes with parallel decrease of glutathione (GSH) [81]. Depletion of liver antioxidant stores and release of hepatic oxygen free radicals may cause organ damage and increase morbidity in malnourished individuals.

Nevertheless, intermittent fasting has been shown to exert beneficial effects similar to caloric restriction, improving risk factors for cardiovascular disease and increasing life span [82]. Both caloric restriction and intermittent fasting may be related to decreased production of free radicals and improved activity of the mechanisms protecting from damaging agents [83]. In addition, the increased resistance to the oxidative stress found during intermittent fasting could contribute to beneficial effects of this regimen [84]. Every-other-day feeding increased maximum life span in experimental animals by decreasing mitochondrial oxidative stress that is independent from insulin/IGF-1 signaling [85]. The intermittent feeding caused decreased ROS production in complex I but not in complex III of the mitochondrial respiratory chain without changes of oxygen consumption.

Short intermittent fasting has an effect similar to caloric restriction on ROS production in mitochondria, with consequent preventive effect on aging.

6.5.4 Physical Activity

However, energy intake is not the only factor influencing oxidative stress in experimental animals or in human life. Physical exercise was found to influence both ROS production and antioxidant defense mechanisms. It was demonstrated that exercise decreases ROS production evaluated by lowered malondialdehyde concentration [86], increases ROS generation confirmed by higher plasma carbonyl derivatives [87], or has a neutral effect [88]. The antioxidant defense system has been activated as measured by increased SOD activities or plasma vitamin E concentration [86, 87, 89]. However, decreased protective effects of antioxidant mechanisms have been observed, too [90, 91]. These controversial results have been reviewed [92]. Changes in oxidative stress were followed during moderate physical exercise by increased median life span, decreased oxidative damage, and prevention of the decline of cytochrome oxidase activity [93]. Regular exercise seems to retard the accumulation of cell damage and physiological dysfunction [94]. Moderate exercise activates DNA repair and increases resistance against oxidative stress [93]. Positive

effects of moderate exercise have been observed in different organs of experimental animals. On the other hand, high-intensity or long-duration exercise accelerates oxidative stress and decreases GSH-to-GSSH ratio [95–97]. The resulting oxidative stress level is dependent on the exercise intensity. Moderate and chronic physical activity may decrease the oxidative stress level, whereas acute and intense exercise accelerates ROS production and may decrease antioxidant enzyme activities [98, 99].

Caloric restriction in combination with moderate physical exercise may be protective against oxidative stress [100]. Lifestyle modification based on both regimens, lower calorie intake and moderate physical activity, has a beneficial outcome in reduced oxidative stress [101]. Both caloric restriction and physical activity may positively influence mitochondrial function, with consequently increased life expectancy [102]. Negative energy balances induced through either caloric restriction or exercise result in improvements in markers of DNA and RNA damage associated with lowered formation of oxidation products [103].

6.5.5 Sex Differences

The link between sex and cardiovascular disease is well documented in both humans and animal studies [104, 105]. The protection of females against cardiovascular complications is attributed mainly to sex hormones [105], but the role of the mitochondrial respiratory chain has been evaluated in the last few years [106]. Clear sex difference in mitochondrial energy metabolism was observed in the rat liver, skeletal muscle, and adipose tissue [107–109], which could be related to different ROS production in males and females. The sexual dimorphism in liver mitochondrial oxidative capacity was unaffected by caloric restriction in rats, with females showing higher mitochondrial functionality and ROS protection than males [110]. The effect of caloric restriction on ROS generation in cardiac muscle tissue was also compared in female and male rats [106]. Cardiac muscle from female rats exhibits lower mitochondrial content, although more differentiated, without any loss of functionality compared with male rats. Caloric restriction decreases mitochondrial hydrogen peroxide production related to lower activity of the respiratory chain complexes I and III but does not contribute to increased antioxidant activity. A greater mitochondrial differentiation with higher oxidative phosphorylation efficiency in female cardiac muscle has been proposed. However, estrogens may have a positive influence, because increased mitochondrial hydrogen peroxide generation was found in the liver and brain from ovariectomized female rats, whereas hydrogen peroxide production

reverted to normal levels when these rats were substituted with estrogens [111]. In another study estrogen-treated rats had antioxidant and hepatoprotective effects [112]. It is of particular interest that both mitochondria and MnSOD are major downstream targets of estradiol signaling [113]. Mitochondria from females were found to contain higher levels of MnSOD than those from males, and their potency to stimulate antioxidant enzymes was higher [114]. However, the effect of estrogen receptor activation to stimulate transcription of mitochondrial MnSOD or GPx is indirect and possibly caused by a signal transduction pathway mediated by MAP kinase, ERK1/2, and NF- κ B (Fig. 6.8).

Estrogen appears to have a protective effect for lipoprotein oxidation in postmenopausal supplemented as compared to nonsupplemented women. A sufficient estrogen level may decrease lipid peroxidation. A sex dimorphism was also demonstrated by high-fat diet-induced changes in lipid oxidation and serum activity of paraoxonase 1 (PON1) [115]. Destabilization of the PON1 association to HDL or direct inactivation of PON1 activity accounted for the decreased serum PON1 activities in female rats and thus for its decreased protective effect. Changes in both ROS generation and scavenger enzymes have to be related to sex differences. They could therefore be taken into account when comparing effects of different interventions on oxidative stress in men and women.

6.6 BIOMARKERS INDICATING MOLECULAR CHANGES IN CALORIC RESTRICTION

The level of oxidative stress depends on both ROS production and the effectiveness of the antioxidant system to scavenge it. The actual oxidative stress is thus created in the respective tissue/organ or in body fluids. Biomarkers may characterize the level of oxidative stress as the actual risk of oxidative damage, and they may elucidate the effect of different treatments or interventions.

Direct measurement of ROS production may be used under experimental conditions, but it brings some difficulties. It can hardly be performed in routine clinical practice in humans. Different products of lipid or protein oxidation may be measured more frequently as indicators of oxidative stress. On the other hand, the antioxidant system represented by enzymatic and non-enzymatic compounds may provide information on protective mechanisms decreasing the oxidative stress level. The impaired protective mechanisms may thus exacerbate oxidative stress and induce downstream activation of NF- κ B with an inflammation cascade.

The evaluation of oxidative stress in experimental conditions and in humans may therefore offer new

insights in biochemistry and pathophysiology of different diseases.

6.6.1 Reactive Oxygen/Nitrogen Species

Mitochondria is the main ROS generator producing superoxide and hydrogen peroxide as the most important oxygen species [3]. Food or caloric restriction as an effective modulator of oxidative stress may reduce ROS generation in mitochondria, as repeatedly demonstrated in several studies after a restricted diet, and lowered ROS could therefore be anticipated. On the other hand, overfeeding induces more ROS compounds, as confirmed by increased levels of lipid or protein oxidation products.

Hydrogen peroxide is usually produced by electron reduction of superoxide, the reaction catalyzed by SOD. Hydrogen peroxide was found in skeletal muscle in a strain of rats characterized by a self-low caloric intake when they consumed a high-fat diet [116]. Caloric restriction (40%) did not alter proton leak or H_2O_2 production in rat liver [117]. Hydrogen peroxide may be measured as a marker of oxidative stress in experimental conditions, but it cannot be directly measured conveniently in clinical studies.

NO is generated from arginine by mtNOS, and subsequently it may be transformed to peroxynitrite. Hydrogen peroxide is the source of harmful hydroxyl radical, whereas peroxynitrite causes nitration of proteins. Nitrotyrosine as an indicator of protein nitration can be used in studies evaluating the role of NO in the oxidative and/or nitrative stress.

6.6.2 Oxidatively Derived Products

Several markers derived by ROS or RNS and indicating the level of oxidative stress can be determined in tissues or in body fluids. Oxidized plasma lipids or the susceptibility of lipids to *in vitro* oxidation and conjugated dienes are examples of such oxidative products [92]. Oxidized low-density lipoprotein (LDL) is strongly immunogenic, and autoantibodies produced against oxidized LDL may be used as a biomarker confirming oxidative changes in LDL molecules.

6.6.3 Malondialdehyde and Thiobarbituric Acid-Reactive Substances

Malondialdehyde as a result of lipid peroxidation can be determined spectrophotometrically using thiobarbituric acid (TBA) or by high-performance liquid chromatography (HPLC). Nonspecific reaction products in biological fluids when TBA reacts with other compounds like saccharides or bilirubin are described as TBA-reactive substances (TBARS).

Caloric restriction was associated with decreased plasma oxidized LDL and lowered malondialdehyde concentrations [118]. Similarly, significantly lower urinary malondialdehyde levels have been found after short fasting in healthy women [119]. A nonsignificant decrease in plasma malondialdehyde after 8 days of very low-calorie diet (600 kcal) in type 2 diabetic patients compared with a significantly decreased malondialdehyde concentration in healthy persons supports the suggestion of a role of insulin resistance in reducing the effect of caloric restriction [120]. Similarly, malondialdehyde was unchanged after dietary caloric restriction in streptozotocin-induced diabetic rats, but a small decrease was observed in nondiabetic animals [121]. Chronic undernutrition in marasmic children increased oxidant status and decreased antioxidant mechanisms both associated with increased malondialdehyde concentrations and lower antioxidant potential [122].

In summary, malondialdehyde may be used as a simple oxidative stress biomarker in both clinical and experimental studies demonstrating the effect of dietary regimen. Its plasma concentration may be increased in the acute phase of experimental conditions as a consequence of oxidative stress activation, whereas its concentration decreases with decreased lipid peroxidation. Differentiation between the changes in the acute and chronic phases is needed to properly evaluate the oxidative stress.

6.6.4 Nitrotyrosine

Nitrotyrosine is created from tyrosine residues reacting with peroxynitrite that is generated from NO and superoxide. Caloric restriction has been associated with decreased nitrotyrosine levels in the brain tissue [123, 124]. Nitrotyrosine concentration was lower in skeletal muscle of rhesus monkeys fed a calorie-restricted diet [125]. This study demonstrated that caloric restriction may attenuate the aging process by reducing oxidative stress. In another study, a low-fat complex-carbohydrate diet reduced nitrotyrosine accumulation in comparison with a high-fat sucrose diet in rats [126]. However, limited results concerning dietary effects on nitrotyrosine changes in humans are available, and follow-up studies will be necessary.

6.6.5 F_2 -Isoprostanes

F_2 -isoprostane is derived from peroxidation of arachidonic acid, and thus it reflects the intensity of lipid peroxidation in biological fluids and tissues. 8-Iso-prostaglandin $F_{2\alpha}$ is a sensitive marker of oxidative stress [127]. Its increased plasma concentration correlated with lipid oxidation in aging of rats [128], whereas caloric restriction

was associated with its decreased plasma as well as liver or kidney concentrations [129]. In addition, F_2 -isoprostanes significantly correlate with 8-oxodeoxyguanosine, an indicator of DNA oxidation. Short-term fasting reduces lipid peroxidation products, as demonstrated by decreased urinary 8-isoprostaglandin $F_{2\alpha}$ and malondialdehyde in healthy women [119]. Combination of exercise with either high-calorie or low-calorie diet was associated with significant decrease of serum F_2 -isoprostanes [130]. Exercise was considered as the main cause of reduced lipid peroxidation, independent of caloric intake. Lifestyle modification characterized by dietary and exercise intervention may ameliorate factors associated with atherosclerosis. A significantly decreased concentration of 8-isoprostaglandin $F_{2\alpha}$ confirms a reduced ROS production [101].

F_2 -isoprostanes as sensitive markers of lipid peroxidation may be used as one of the best indicators of oxidative stress. The F_2 -isoprostane concentrations should be assessed simultaneously in plasma and urine because some divergencies bring difficulties in interpretation of data [131].

6.6.6 8-Hydroxydeoxy-guanosine

Mitochondrial DNA damage is related to accelerated ROS production. 8-Hydroxydeoxy-guanosine, a marker of DNA damage due to oxidative stress, correlates with F_2 -isoprostanes or malondialdehyde, markers of ROS generation [129]. The association of increased oxidative stress with mtDNA damage was proved in mice heterozygous for the *SOD2* gene encoding mitochondrial MnSOD [132]. In these animals increased 8-hydroxydeoxy-guanosine production was confirmed. Caloric restriction was associated with reduced ROS production and decreased DNA damage as measured by 8-hydroxydeoxy-guanosine [129]. However, short-term fasting, despite decreased lipid peroxidation, did not reduce 8-hydroxydeoxy-guanosine [119].

Concentration of 8-hydroxydeoxy-guanosine does not always correlate with ROS generation, and DNA damage should be evaluated separately when changes in oxidative stress are observed.

6.6.7 Scavenger Enzymes

Enzymes detoxifying free oxygen radicals and related molecules play an important role in the balance between ROS production and elimination. Enzyme activity significantly determines the intracellular level of oxidative stress; however, the final results frequently depend on cooperation between several enzymes catalyzing a cluster of reactions from ROS intermediates to stable molecules. The oxidative stress evaluation should be based on the estimation of several enzyme activities influencing cascade

reactions of intermediate ROS molecules. Genetic and environmental factors may influence enzyme activities and their efficiency to catalyze the appropriate reactions.

6.6.7.1 Superoxide Dismutase The superoxide generated as the most common ROS is removed by two types of superoxide dismutase, cytoplasmic Cu,Zn-SOD and mitochondrial MnSOD. Overeating associated with high energy intake causes dramatic increase of substrates for metabolic pathways, subsequently accelerating the mitochondrial respiratory chain. High superoxide generation is often combined with increased SOD activity. Reduced energy intake produces fewer free radicals, leading to less oxidative damage. It could therefore be interesting to learn whether caloric restriction may influence SOD activity. Experimental studies using calorie-restricted diets showed increased [133], decreased [134], or not changed [121, 135, 136] SOD activity. In another study, MnSOD mRNA was increased after short-term caloric restriction but without elevation of protein level [137]. The increase of myocardial SOD was explained by a synergistic action of olive oil added to a calorie-restricted diet [138]. Short-term very low-calorie diet induced a significant increase of SOD activity in both diabetic and control subjects [120]. Aging was associated with higher ROS generation, but combination with food restriction led to attenuated aging. Similarly, caloric restriction was associated with improved endothelial dysfunction during vascular aging [139]. This effect was caused by an improved ratio of nitric oxide synthase isoforms (iNOS/eNOS) in consequence to reduced oxidative stress. The SOD activity was found to have a key role in this balance because the removal of superoxide may further improve the availability of NO. In another study, the SOD activities, while increased with aging, were attenuated by food restriction [140].

MnSOD as a sole mitochondrial SOD in mammals and birds has a dominant effect on ROS removal in mitochondria. Its expression and activity in the brain correlated with life span in vertebrates, whereas no such relationship was found in the case of cytosolic Cu,ZnSOD or GPx [141]. Hence, MnSOD seems to be an important predictor of the mitochondrial defense system modulating the availability of superoxide for its harmful effects.

6.6.7.2 Catalase Catalase is a ubiquitous heme protein enzyme that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen. The enzyme is therefore localized in a close downstream position relative to SOD. This enzyme relationship may predict similar changes of their activities [133–135]. Caloric restriction has been associated with increased [133], decreased, or unchanged catalase activities. Elevation of SOD activity sometimes occurs together with

decreased catalase activity [134] when increased GPx activity is observed. An oscillatory and frequently inverse relationship between the activities of catalase and GPx has been proposed [134].

6.6.7.3 Glutathione Peroxidase GPx detoxifies peroxides with reduced form of glutathione (GSH) acting as an electron donor producing GSSG. Different GPx iso-enzymes have been described in cytosol or in extracellular space [142]. GPx may share the same substrate, hydrogen peroxide, with catalase. Accelerated ROS production has been associated frequently with increased GPx activity [133, 135]. The enzyme activity was depressed in diabetic animals feeding “ad libitum,” but it was increased in both diabetic and control animals by caloric restriction [135]. A significant decrease in GPx activity may suggest either enzyme inactivation by increased ROS or decreased substrate (GSH) availability. In such cases an oscillatory elevation in the catalase activities has been observed [135]. Significantly increased GPx activity together with increased GSH-to-GSSG ratio in kidney was found after caloric restriction in mice [136]. These changes demonstrate increased antioxidant capacity as a consequence of functional impact of dietary regimen.

6.6.7.4 Glutathione Reductase Oxidized glutathione (GSSG) is recycled to reduced form GSH by glutathione reductase (GR), and GSH may be reused in antioxidant defense. GR therefore has a pivotal role in regeneration of GSH. GR and GPx activities were increased in experimental animals after caloric restriction [134]. Such changes increase the capacity of antioxidative system and reduce the level of oxidative stress by caloric restriction. However, other experiments did not demonstrate any changes in liver GR activity in rats affected by 30% to 40% restriction in caloric intake [143, 144]. Improved antioxidative state was associated with changes of other components of oxidative stress (i.e., lowered ROS production and increased activities of other scavenger enzymes).

6.6.7.5 Paraoxonase 1 Paraoxonase 1 (PON1), a sex-dependent enzyme specifically associated to high-density lipoproteins (HDL), has been shown to hydrolyze lipid peroxides present in low-density lipoproteins (LDL). Its activity is sensitive to different diets and other factors as well [145]. Short-term dietary restriction was associated with decreased serum PON1 activity, but no changes were found in liver PON1 mRNA levels [145]. Early and prompt increase in rat serum PON1 activity was induced already during the first hours of fasting [146]. This was followed by progressive decrease of PON1 activity and a profound decrease of liver PON1 mRNA levels. Decreased GPx activity was found during fasting,

possibly due to depleted liver glutathione concentration and elevated lipid peroxide levels. Deleterious effect of prolonged fasting is therefore explained by accelerating oxidative stress together with decreased antioxidant mechanisms. Other factors like acidosis or ketone bodies in prolonged fasting may further contribute to decreased PON1 activities by accelerating oxidative stress [147]. Early increase of oxidative stress during the initial hours of fasting may stimulate the antioxidant response of PON1 by increasing its activity [148].

The above results demonstrate that the low-intensity oxidative stress provided by repeated short periods of fasting would elicit a defense response that may enhance protective mechanisms, for example, antioxidative enzymes [145].

6.6.8 Nonenzymatic Scavengers

The human body contains endogenous compounds that have a protective role and act as antioxidants. Both low (glutathione, ascorbic acid, α -tocopherol, coenzyme Q, lipoic acid, uric acid, bilirubin, etc.)- and high (transferin, ceruloplasmin, albumin, metallothioneins, or chaperones)-molecular-weight compounds have a significant role in maintaining ROS homeostasis. Several compounds have been used in evaluating antioxidative state, such as glutathione, ascorbic acid, etc., but some of them are frequently determined in daily routine practice without any relationship to oxidative stress (uric acid, bilirubin, albumin, etc.).

6.6.8.1 Glutathione Tripeptide glutathione (γ -glutamyl-cysteinyl-glycine) is an important redox buffer inside cells. GSH is required to suppress oxidative stress and to maintain the normal reduced state of the cell. Recycling of reduced (GSH) and oxidized (GSSG) forms is regulated by ROS production and the antioxidant enzymes GPx and GR. The GSH-to-GSSG ratio and antioxidant enzyme activities have been used to assess the oxidative stress level. A lower ratio is associated with accelerated oxidative stress when recycling by GR is diminished. Overfeeding or feeding “ad libitum” in animal experiments decreases GSH concentration, whereas GSSG levels are increased [134, 135]. Caloric restriction is able to increase GSH levels and decrease GSSG; the GSH-to-GSSG ratio is therefore increased. Glutathione redox potential in mitochondria of different organs becomes less negative and/or more prooxidant with aging [149, 150]. Administration of a calorie-restricted diet may attenuate GSH decline and therefore may retard the age-related degenerative processes caused by accelerated oxidative stress.

GSH and GSH/GSSG determination may be used as reliable markers of the oxidative stress. Their correlation

with scavenger enzyme activities offers important information on defense mechanisms against oxidative stress under different conditions in health and disease.

6.6.8.2 Ascorbic Acid Ascorbic acid reduces organic and inorganic radicals. It is involved in regeneration of tocopheryl radical to α -tocopherol, a reaction producing ascorbyl radical as a source of dehydroascorbic acid. Ascorbic acid regeneration has important role in reducing the “pro-oxidative” properties of dehydroascorbic acid. This ambivalent role of ascorbic acid depending on both ROS generation and antioxidative mechanisms may be the source that worsens the oxidative state either inside or outside cells. Regeneration of ascorbic acid and other compounds cycling between the reduced and oxidized forms occurs via the electron transport process (Fig. 6.9).

Ascorbic acid levels may be decreased by aging or generally by accelerated oxidative stress [150]. Caloric restriction attenuating the aging process may also slow down the decline in ascorbic acid level in rat retina. Although short-term very low-calorie diet induced ascorbic acid increase in healthy persons, this was not found in type 2 diabetic patients [120]. Such a difference could be caused by antioxidative abnormalities in diabetes, although data are lacking for more information.

6.6.8.3 α -Tocopherol α -Tocopherol is a lipophilic antioxidative compound preserving membrane against lipid peroxidation. It transforms fatty acid peroxy radicals to hydroperoxides that are further transformed by GPx. Tocopherol is converted to tocopheryl radical that may be partly reduced by ascorbic acid.

Aging is associated with increased oxidative stress characterized by increased lipid peroxidation markers, protein carbonyls, or nitrotyrosine and lower antioxidant defenses. A positive association was found between plasma SOD or α -tocopherol and survival in a longitudinal study [151]. The highest survival was observed in patients with high serum α -tocopherol and low plasma malondialdehyde concentrations [151]. Caloric restriction attenuates a decline of α -tocopherol content in

plasma membrane caused by aging [152, 153]. Restriction of calorie intake was associated with decreased markers of oxidative stress and increased enzyme activities protecting cells against age-related oxidative stress [153]. However, the mitochondrial α -tocopherol content was diminished by calorie-restricted diet in rat [154]. Short-term very low-calorie diet was associated with a decrease of serum α -tocopherol levels, but its content in plasma membranes was not determined [120]. The α -tocopherol changes may contribute to accelerated oxidative stress when production of tocopheryl radicals is not sufficiently reduced. The isolated supplementation by α -tocopherol under conditions with accelerated oxidative stress may induce adverse reactions and further worsen the oxidative state [155].

6.6.8.4 Ubiquinone (Coenzyme Q) Coenzyme Q (ubiquinone) is another lipophilic antioxidant that cooperates with α -tocopherol in plasma membranes. It influences the transmembrane redox system and protects cell membrane against lipid peroxidation. Coenzyme Q has three functions in mitochondria: (a) transfer of reducing equivalents in the electron transport chain (Fig. 6.1), (b) generation of superoxide anion radical, and (c) free radical quenching. As with α -tocopherol, coenzyme Q content declines with aging. Dietary supplementation with coenzyme Q augmented endogenous mitochondrial content in various tissues, but it had no significant effect on the main antioxidant defenses or prooxidant generation [156, 157]. Caloric restriction increased coenzyme Q level and attenuated aging [124, 152, 154].

6.6.8.5 Other Markers The association of fluorescent oxidation products with several indicators of oxidative stress suggests that this measure could be a global marker of oxidative stress [158]. There are no data evaluating these products in caloric restriction. However, fluorescent products highly correlated with oxidative stress associated with smoking.

Different biochemical markers have been used to create the OXY-SCORE index [159], which reflects

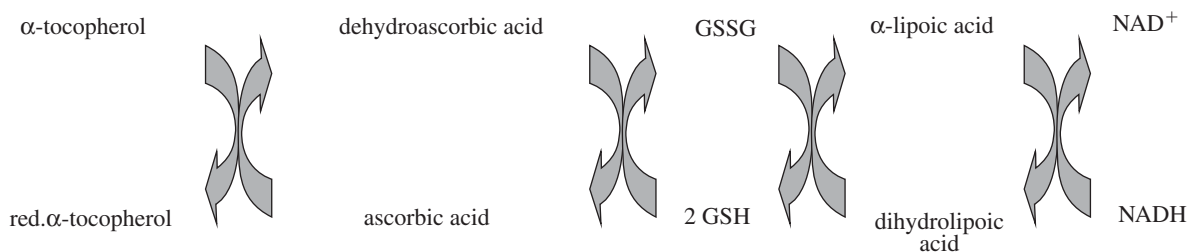


Fig. 6.9 Cycling of antioxidants and their interrelationship.

both oxidant and antioxidant markers. The damage score is characterized by plasma free and total malondialdehyde, glutathione disulfide/reduced form ratio, and urine isoprostanes, whereas the protection score is based on reduced glutathione (GSH), α - and γ -tocopherol levels, and antioxidant capacity. The index calculated as difference between damage and protection scores is related to age and sex, but more data are needed in different conditions.

6.7 CONCLUSION

Oxidative stress is an important process that modifies cellular reactions in both health and disease. It stimulates intracellular pathways promoting development of various diseases but also initiates reparative reactions (e.g., wound healing). Homeostatic mechanisms may sustain oxidative stress under control. Advanced production of ROS or RNS activates protective mechanisms that may eliminate the effects of deleterious compounds when their capacity is sufficient. In the case of insufficient power of these protective mechanisms, homeostasis is impaired.

Increased caloric intake causes overproduction of ROS, especially in the respiratory chain of mitochondria. The substrate overload increases the amount of superoxide generated and then accumulated in the mitochondrial matrix. Insufficient or exhausted capacity of the antioxidant system enables the development of harmful effects in mitochondria and in the cell as well. It may be therefore important to learn whether caloric restriction would be beneficial for ROS homeostasis.

Caloric restriction and prolonged fasting are different conditions. The lower energy consumption or decreased protein (methionine) intake attenuates ROS generation, and thus oxidative products may be decreased. Different changes in antioxidant enzymes involving increased, unchanged, or decreased activities may be dependent on different experimental conditions and on individual responsiveness caused by genetic and epigenetic factors. Increased glutathione (GSH) level may reflect attenuated oxidative stress because greater reducing capacity is present, whereas advanced oxidative stress is frequently associated with decreased GSH and decreased GSH-to-GSSG ratio. The results of oxidative markers depend on timing during the experimental procedure. The initial changes characterized by stimulated ROS production (i.e., increased malondialdehyde concentration) may be followed by depressed ROS generation (decreased malondialdehyde or F₂-isoprostane levels). Short or prolonged exposure to caloric restriction may cause different responses in ROS generation and antioxidative mechanisms. The hormetic role of dietary products

increasing cellular stress resistance brings new insights into physiology of oxidative stress regulation [160]. Reduced energy consumption by controlled caloric restriction or intermittent fasting increases life span and protects various tissues against oxidative stress, partly by hormetic mechanisms. Hormesis is based on a biphasic dose-response relationship in which a low dose stimulates and a high dose inhibits protective mechanisms. Thus calorie-restricted diets and their composition may significantly improve ROS homeostasis both in single cells and in the whole body.

On the other hand, prolonged fasting stimulates ROS production and does not provide any benefit. Oxidative products are usually increased, whereas the antioxidant system may be differently modified. More data on molecular mechanisms and regulation of oxidative stress under overnutrition and caloric restriction are still necessary.

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PATHOGENESIS OF NEURODEGENERATIVE DISEASES: CONTRIBUTION OF OXIDATIVE STRESS AND NEUROINFLAMMATION

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7.1 INTRODUCTION

Neurodegenerative diseases are characterized by slow death of specific neuronal populations and synapses in brain and spinal cord. In neurodegenerative diseases, the degenerating neuronal population modulates thinking, skilled movements, decision making, cognition, and memory [1, 2]. These diseases are characterized by chronic cellular processes that lead to cumulative insults to the nervous system. Neurodegenerative diseases include Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS), and prion diseases. In AD, neurodegeneration mainly occurs in the nucleus basalis. In PD, neuronal demise takes place in the substantia nigra. Neurodegeneration of striatal medium spiny neurons contributes to the pathogenesis of HD, and ALS is characterized by the death of motor neurons in the brain and spinal cord. Although it is not clear when the neurodegenerative process actually starts and how long it takes for neuropathological symptoms to appear, it is becoming increasingly evident that old age, positive family history, unhealthy lifestyle, and exposure to toxic environment may contribute to the pathogenesis of neurodegenerative diseases (Fig. 7.1) [3–5]. As stated above, most neurodegenerative diseases are accompanied by the premature and slow death of specific neuronal populations, increase in oxidative stress, and

neuroinflammation. These processes contribute to the modulation of brain function through not only alterations in levels of phospholipid, sphingolipid, and cholesterol-derived lipid mediators but also accumulation of misfolded and aggregated proteins, which threaten neuronal viability [6]. Despite the important differences in clinical manifestation and progressive cell loss of specific neuronal populations in a specific region, neurodegenerative diseases share some common features, such as excitotoxicity, synaptic dysfunction, and the accumulation of intracellular or extracellular cerebral deposits of misfolded protein aggregates with a β -sheet conformation, such as β -amyloid ($A\beta$) in AD, α -synuclein in PD, huntingtin in HD, and mutation in Cu/Zn-superoxide dismutase (SOD) in ALS (Table 7.1) [6]. In addition to the above alterations, neurodegenerative diseases also share some terminal neurochemical common processes including excitotoxicity, oxidative stress, and inflammation [7]. It remains controversial whether these processes are the cause or the consequence of a neurodegenerative process [8, 9]. The onset of neurodegenerative diseases is often subtle and usually occurs in middle to late life, and their progression depends not only on genetic but also on environmental factors [1]. Although a number of indices of oxidative stress and neuroinflammation such as protein oxidation and nitrosylation, lipid peroxidation, DNA oxidation, and 3-nitrotyrosine formation as well as diminished levels of antioxidants such as SOD

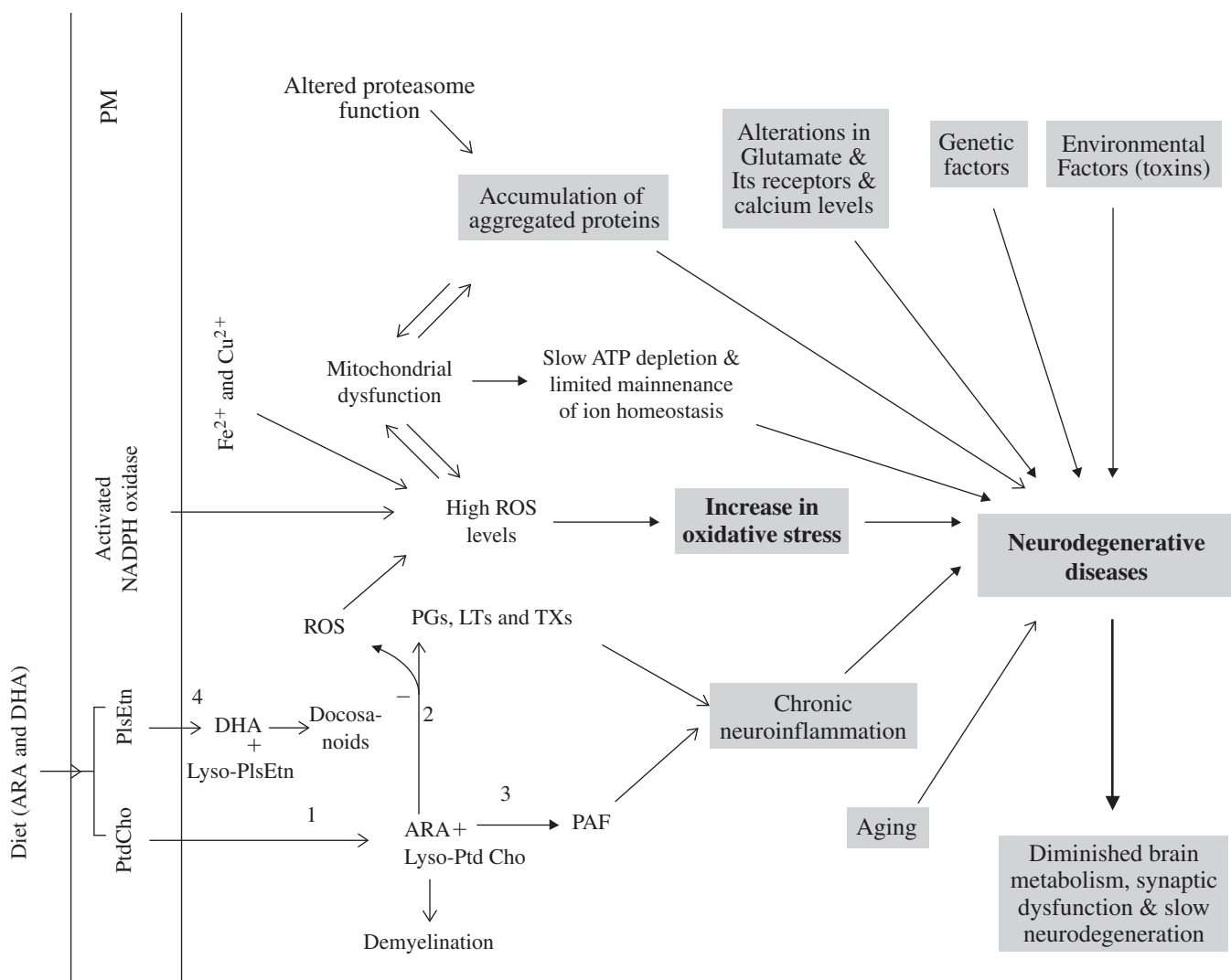


Fig. 7.1 Factors modulating neurodegenerative diseases: cytosolic phospholipase A₂ (1); cyclooxygenases and lipoxygenases (2); acetyltransferase (3); plasmalogen-selective phospholipase A₂ (4). ARA, arachidonic acid; DHA, docosahexaenoic acid; PtdCho, ARA-containing phosphatidylcholine; PlsEtn, DHA-containing ethanolamine plasmalogen; lyso-PtdCho, lyso-phosphatidylcholine; lyso-PlsEtn, lysoplasmalogen; ROS, reactive oxygen species; PGs, prostaglandins; LTs, leukotrienes; TXs, thromboxanes; PAF, platelet-activating factor. DHA-derived docosanooids inhibit the generation of eicosanoids (PGs, LTs, and TXs).

and increased expression of proinflammatory cytokines have also been reported to occur in neurodegenerative diseases [6, 10], very little information is available on timing, selective cellular vulnerability, and progression of neurodegenerative diseases.

7.2 OXIDATIVE AND NITROSATIVE STRESS IN NEURODEGENERATIVE DISEASES

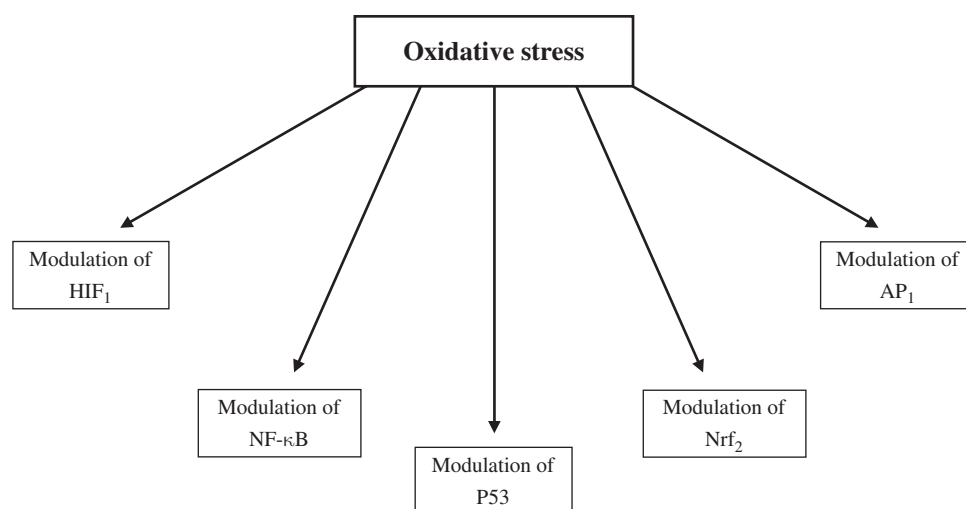
Oxidative stress is defined as a cytotoxic process that occurs in the cell when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS), which are atoms or molecules possessing one or more unpaired

electrons in the outer orbit and therefore prone to react chemically [11]. Thus oxidative stress is characterized by a major increase in the amount of oxidized cellular components. ROS include superoxide anions ($O_2^{\bullet-}$), hydroxyl ($\bullet OH$), alkoxyl (RO^{\bullet}), and peroxy radicals (ROO^{\bullet}), and hydrogen peroxide (H_2O_2). The major sources of ROS include the mitochondrial respiratory chain, xanthine oxidase, myeloperoxidase in cytoplasm, oxidation of arachidonic acid (ARA) by cyclooxygenase (COX), lipoxygenase (LOX), and epoxygenase (EPOX) in cytoplasm, and NADPH oxidase in plasma membranes. NADPH oxidase generates superoxide radical by the one-electron reduction of oxygen, using NADPH as the electron donor. In neurons and neuroblastoma cells,

TABLE 7.1 Status and levels of lipid mediators, excitotoxicity, oxidative stress, and neuroinflammation in neurodegenerative diseases

Parameter	AD	PD	HD	ALSALS
Neurodegeneration site	Nucleus basalis and hippocampus	Substantia nigra	Striatum	Motor neurons in anterior spinal cord
Glu/Glu receptor levels	Altered	Altered	Altered	Altered
Ca ²⁺ levels	Increased	Increased	Increased	Increased
Cytokine expression	Increased	Increased	Increased	Increased
Oxidative stress	Increased	Increased	Increased	Increased
Neuroinflammation	Increased	Increased	Increased	Increased
Aggregated protein accumulation	A β levels increased	α -Synuclein	Huntingtin increased	Cu ²⁺ /Zn ²⁺ SOD increased
Mitochondrial function	Abnormal	Abnormal	Abnormal	Abnormal
4-HNE	Increased	Increased	Increased	Increased
Isoprostanes	Increased	Increased	Increased	Increased
Ceramide	Increased	Increased	Increased	Increased
Hydroxycholesterols	Increased	Increased	Increased	Increased
Apoptosis	Increased	Increased	Increased	Increased
BBB permeability	Abnormal	Abnormal	Abnormal	Abnormal

Information adapted from references [4, 6, 30, 72]

**Fig. 7.2** Modulation of transcription factors by oxidative stress.

NADPH oxidase-mediated ROS synthesis is implicated in redox signaling mechanisms, which are modulated by the aging process in brain [12, 13]. The ability of NADPH oxidase inhibitors to ameliorate ROS-mediated cytotoxicity provides strong support for the role of this enzyme in regulation of neuronal excitatory activity. In the presence of metal ions, such as Fe²⁺ and Cu²⁺, H₂O₂ is also transformed into hydroxyl radical (\cdot OH) through the Fenton reaction. Hydroxyl radicals can attack polyunsaturated fatty acids in neural membrane phospholipids, forming the peroxy radical (ROO \cdot), and then propagate the chain reaction of lipid peroxidation. ROS production plays an important role in cell signaling. During normal

aerobic metabolism, ROS generation is kept under tight control through the activities of antioxidant defense systems. The disruption of this tight control by high ROS levels results in oxidative stress. Low levels of ROS are needed for fundamental cellular functions, such as growth and adaptation responses. Specifically, ROS are implicated in mitogen-activated protein kinase (MAPK) pathways, which induce activation of various nuclear transcription factors, such as nuclear factor (NF)- κ B, activator protein-1 (AP-1), hypoxia-inducible factor (HIF)-1 α , and sterol regulatory element binding proteins (SREBPs) [14] (Fig. 7.2). These transcription factors occur in cytoplasm. Generation of high ROS in neural

cells facilitates their translocation from cytoplasm to the nucleus, where they facilitate the expression of proinflammatory enzymes, cytokines, chemokines, growth factors, cell cycle regulatory molecules, adhesion molecules, and antiinflammatory molecules (Fig. 7.3). NF- κ B is associated with initiation and orchestration of inflammatory pathways through the production of TNF- α , IL-1 β , and adhesion molecules. AP-1 is involved in increased expression of adhesion molecules and inflammatory cytokines. HIF-1 α activates a broad range of genes protecting cells against hypoxia. Induction of HIF-1 α during oxidative stress represents the response

of neurons suffering from hypoxic/ischemic insult. In addition, HIF-1 α binds to p53 and also activates the expression of various genes including Bax (a proapoptotic member of the Bcl-2 family) [15]. Bax inactivates Bcl-2 by forming a heterodimer. The balance between levels of bcl-2 and bax can serve as an indicator of cell survival or death [16]. The transcription factor NF-(erythroid-derived 2)-related factor (Nrf2) is associated with regulation of basal and inducible expression of numerous antioxidant stress genes and plays an important role in neuroprotection against oxidative stress in many animal models of neurodegenerative diseases.

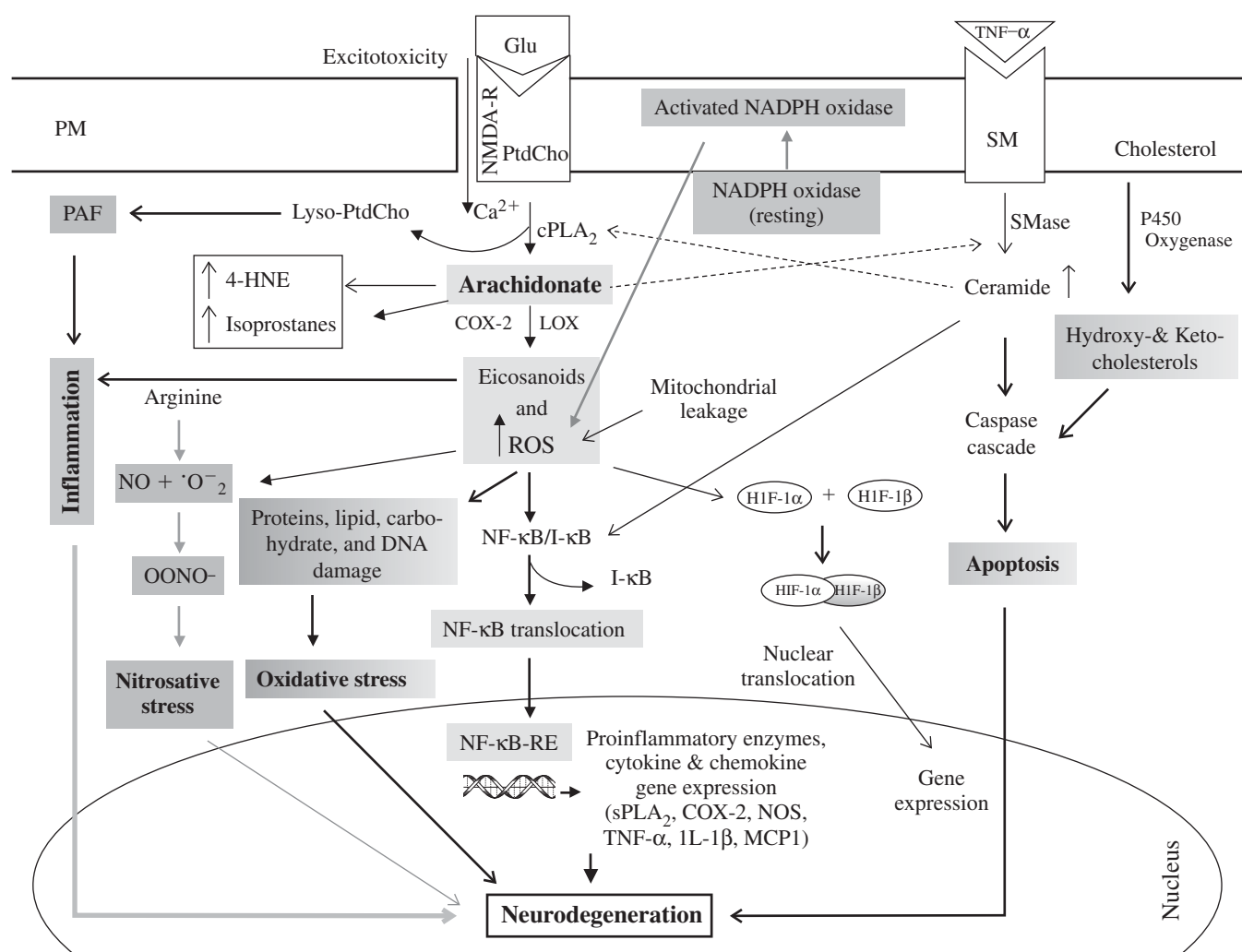


Fig. 7.3 Generation of lipid mediators and interactions between excitotoxicity, oxidative stress, and neuroinflammation in neurodegenerative diseases. cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase-2; LOX, lipoxygenase; NOS, nitric oxide synthase; SMase, sphingomyelinase; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin 1 β ; lyso-PtdCho, lyso-phosphatidylcholine; ROS, reactive oxygen species; HIF-1, hypoxia-inducible factor-1; 4-HNE, 4-hydroxynonenal; NO, nitric oxide; OONO⁻, peroxynitrite; PAF, platelet-activating factor. Activation of NF- κ B by ROS leads to its translocation to the nucleus, where it facilitates the transcription of proinflammatory enzymes (sPLA₂, COX-2, NOS, and SOD) and proinflammatory cytokines (TNF- α and IL-1 β). These cytokines upregulate activities of cPLA₂ and sPLA₂ through a positive loop mechanism in cytoplasm and neural membranes. Furthermore, 4-HNE and isoprostanes promote neurodegeneration. Red arrows indicate increase in levels of metabolites. (See color insert.)

In neurodegenerative diseases enhanced ROS levels contribute to neuronal membrane damage not only by attacking neural cell membrane components (polyunsaturated fatty acids, sulfhydryl groups of proteins, bases of nucleic acids, and carbohydrates), but also by altering activities of various transcription factors (NF- κ B, AP-1, HIF-1, and Nrf2). It is important to stress that ROS-mediated damage to the above neural membrane components is cumulative and not amenable to repair, particularly in postmitotic cells such as neurons. Reactions between ROS and cellular components can alter cell membrane properties such as fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, and DNA damage eventually leading to neural cell apoptosis, a type of cell death that commonly occurs in neurodegenerative disease [6]. In addition, oxidative damage to mitochondrial DNA (mtDNA) not only leads to mutation but also triggers proapoptotic protein release from mitochondria into the cytoplasm, further contributing to impairment of cell viability.

In neurodegenerative diseases, excessive generation of nitric oxide (NO) due to the overactivation of NMDA receptor in neurons (excitotoxicity) [17] or induction of nitric oxide synthase (NOS) in the neighboring glia (microglial cells and astrocytes) results in generation of NO. The covalent reaction between NO and thiol groups of specific protein is called *S*-nitrosylation. Nitrosylation modifies function of many proteins by altering the hydrophobicity, hydrogen bonding, and electrostatic properties within the targeted protein. *S*-nitrosylation also contributes to protein misfolding of many proteins including redox-sensitive enzyme, disulfide isomerase, and dynamin-related protein 1 (SNO-Drp1) [18–20] in the endoplasmic reticulum, promoting neurodegeneration [21]. Nitrosylation of perkin, which is catalyzed by E3 ubiquitin ligase, may promote the pathogenesis of PD. Inhibition of nitrosylation produces neuroprotective effects [19, 21, 22].

Brain tissue from AD, PD, and ALS contains many nitrated proteins [23–27]. Recent studies using proteomics in brain from mild cognitive impairment subjects and AD patients indicate that many other proteins like peroxiredoxin 2, triose phosphate isomerase, glutamate dehydrogenase, neuropolypeptide h3, phosphoglycerate mutase1, H⁺-transporting ATPase, α -enolase, and fructose-1,6-bisphosphate aldolase are also covalently modified through nitration [28]. In addition, interaction between NO and superoxide anion leads to the formation of the powerful oxidant species peroxynitrite (ONOO⁻). The activation of NAD⁺-consuming enzyme poly(ADP-ribose) polymerase-1 (PARP-1) is another likely mechanism for NO-mediated energy failure and neurotoxicity [29, 30]. NO also binds to

cytochrome *c* oxidase and is able to inhibit cell respiration in a process that is reversible and in competition with oxygen. This action leads to the release of more superoxide anion from the mitochondrial respiratory chain.

In neural membranes, the hydrophobic environment maximizes the formation of reactive nitrogen species (RNS) [31, 32]. In addition to proteins, RNS react with unsaturated fatty acids (e.g., oleic acid) generating nitrooleic acid, a highly reactive electrophilic compound that can modulate a variety of cellular targets, including thiol residues and peroxisome proliferator-activated receptor (PPAR) γ [33–35].

Unlike 4-hydroxynonenal (4HNE), which is generated from the peroxidation of free arachidonic acid [36], nitrooleic acid remains esterified in the neural membrane phospholipids [37]. It is proposed that esterified nitrated fatty acids represent a sink of bioactive mediators, which are produced during nitrative stress leading to cellular dysfunction after release from the membrane by phospholipase A₂ [34]. Free nitrooleic acid is a stimulator of somatosensory and visceral nociceptors. It acts through the selective and direct activation of transient receptor potential cation channel, subfamily A1 (TRPA1) channels in a concentration-dependent manner [32]. Although the role of nitrooleic acid in neurodegenerative diseases is not fully understood, several studies indicate that 9- and 10-nitro-9-*cis*-octadecenoic acid is a potent ligand for PPARs at physiological concentrations [32, 37]. PPAR γ agonists prevent A β neurotoxicity in hippocampal neurons. In addition, based on concentration-response analysis in both neurons and hTRPA1-HEK cells, it is suggested that nitrooleic acid is the most potent endogenous TRPA1 agonist. Emerging evidence suggests that nitrated fatty acids comprise a class of NO-derived, receptor-dependent, cell signaling mediators that act within physiological concentration ranges. Their levels are increased in neurodegenerative diseases, which are accompanied not only by a higher degree of ROS and NO production but also by diminished functions of mitochondria, endoplasmic reticulum, and the proteasome system, which are responsible for the maintenance of the normal protein homeostasis of neural cells [29, 30, 38].

7.2.1 Oxidative Stress-Mediated Damage to Neural Membrane Components in Neurodegenerative Diseases

It is well known that neural membranes are composed of phospholipids, sphingolipids, cholesterol, and proteins [6]. Arachidonic acid (ARA) and docosahexaenoic acid (DHA) are major polyunsaturated fatty acids found in neural membrane glycerophospholipids. Under normal conditions, isoforms of phospholipase A₂ (PLA₂) liberate ARA and DHA. Small amounts of ARA and DHA are

oxidized to eicosanoids (prostaglandins, leukotrienes, thromboxanes, and lipoxins) [39] and docosanoids (resolvins, neuroprotectins, and maresins) (Fig. 7.4) [40–42], respectively, by cyclooxygenases (COXs) and lipoxygenases (LOXs), whereas the majority of ARA and DHA are reincorporated into neural membrane phospholipids [39, 43].

Lysophosphatidylcholine (lyso-PtdCho), the other product of PLA₂-catalyzed reaction, is converted into platelet-activating factor (PAF), another proinflammatory lipid mediator. In neurodegenerative diseases, stimulation of PLA₂ isoforms (Table 7.1) and accumulation of ARA not only lead to the uncoupling of oxidative phosphorylation, resulting in mitochondrial dysfunction [4], but also trigger an uncontrolled “arachidonic acid cascade.” This sets the stage for increased production of ROS that enhance oxidative stress [6]. Furthermore, nonenzymic peroxidation of ARA and DHA produces 4-HNE and 4-hydroxyhexenal (4-HHE), respectively. 4-HNE is a nine-carbon α , β -unsaturated aldehyde (Fig. 7.5), which is one of the major end products of ARA peroxidation and an important mediator of neural cell damage because of its ability to covalently modify proteins, which are important cellular functions [30, 44, 45]; 4-HNE reacts with lysine, cysteine, and histidine residues in proteins [44]. The C3 position of 4-HNE is a

highly reactive site that undergoes a Michael addition reaction with cellular thiols and hence readily forms adducts with glutathione or proteins containing thiol groups. 4-HNE not only inhibits key membrane proteins including glucose transporter, glutamate transporter, and sodium, potassium ATPases [45–49], but also inhibits rat brain mitochondrial respiration, blocks neurite outgrowth, disrupts neuronal microtubules, and modifies cellular tubulin, which may contribute to the cytoskeletal changes in neurons undergoing a neurodegenerative process [50, 51]. Collective evidence suggests that 4-HNE triggers multiple signaling cascades that variably affect cell growth, differentiation, and apoptosis [52]. 4-HNE inhibits DNA and RNA synthesis. Nonenzymic oxidation of DHA generates 4-HHE (Fig. 7.5). Like 4-HNE, 4-HHE reacts readily with nucleophiles such as thiols and amines, while the carbonyl group forms Schiff bases with amino groups such as the N-termini of proteins and the ϵ -amino group of lysine. 4-HHE binds to proteins. These carbonyl derivatives are potential markers of oxidative stress [30, 53].

In neurodegenerative diseases nonenzymic oxidation of esterified ARA also results in the generation of isoprostanes, which are prostaglandin-like mediators (Fig. 7.5) [54, 55]. The molecular mechanism of F₂-isoprostanes involves the formation of positional

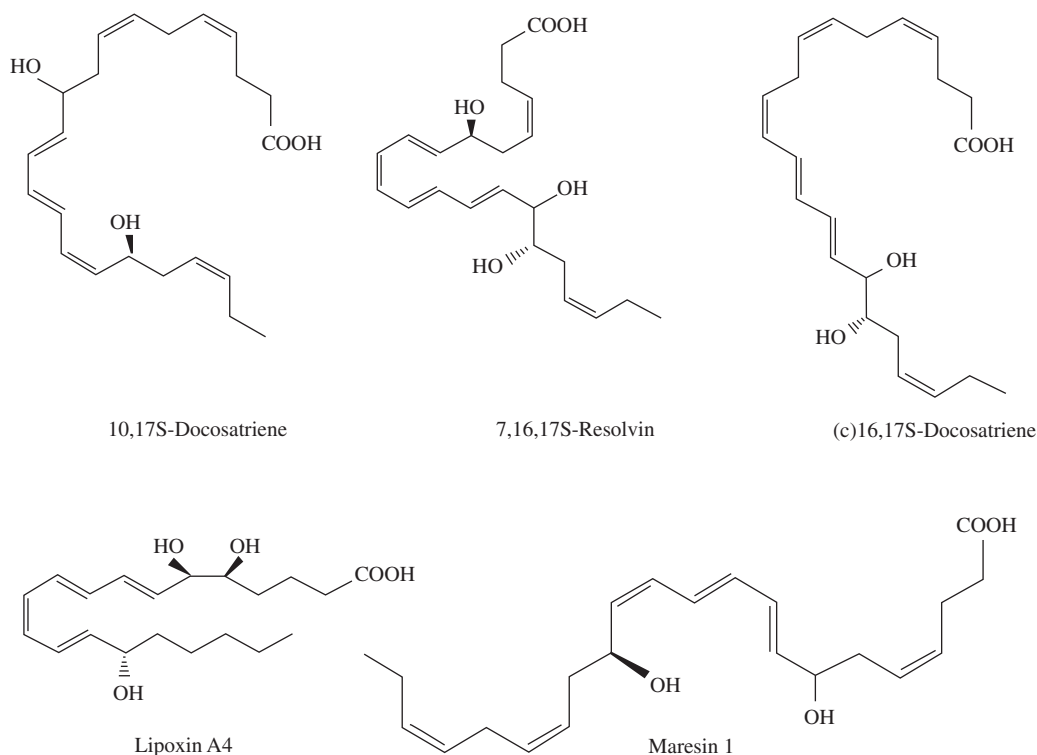


Fig. 7.4 Chemical structures of DHA-derived lipid mediators: 10,17S-docosatriene; 7,16,17S-resolvin; 16,17S-docosatriene; lipoxin A4 (this mediator is derived from ARA); and Maresin 1.

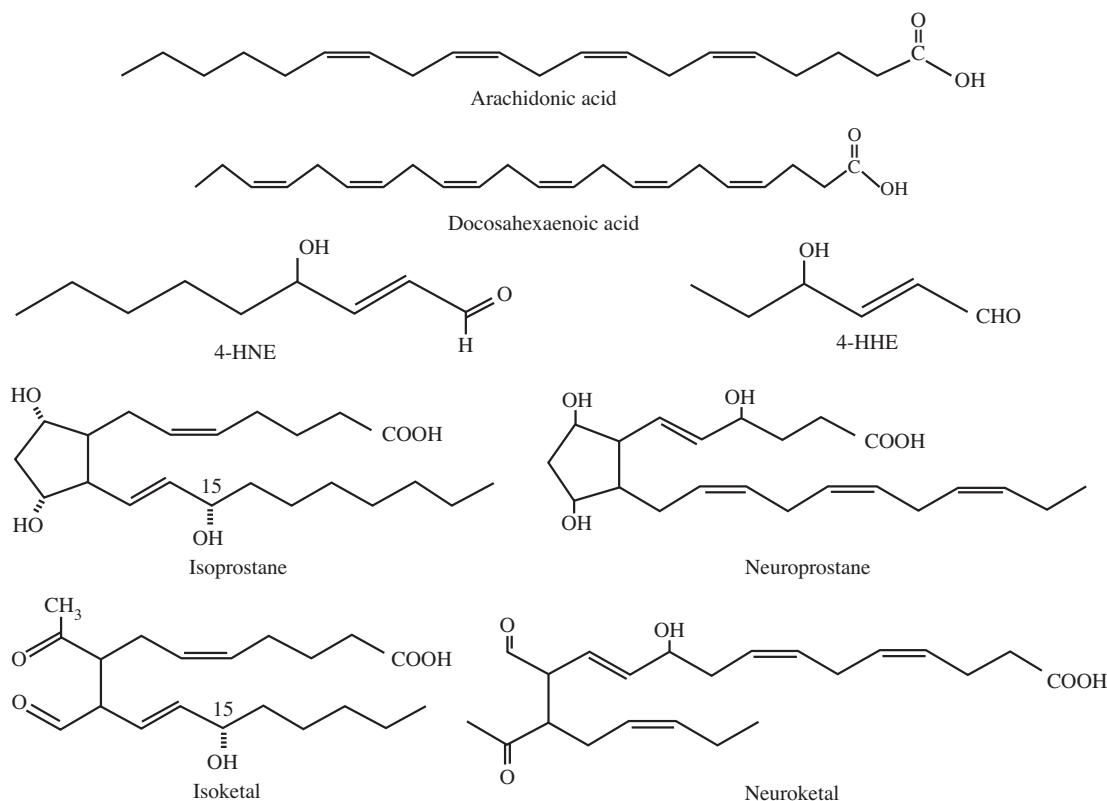


Fig. 7.5 Chemical structures of nonenzymic lipid mediators derived from ARA and DHA.

peroxyl radical isomers of ARA, which undergo endocyclization to form PGG_2 -like compounds. These compounds are reduced to PGF_2 -like compounds. F_2 -isoprostane (F_2 -IsoP) is subsequently released in free form by the action of PLA_2 [56–58]. F_2 -IsoP modulates the p38 MAPK pathway during monocyte adhesion [59]. Isoprostane-mediated monocyte adhesion does not depend on VCAM-1 but involves protein kinases, such as protein kinase A and mitogen-activated protein kinase kinase 1. Thus F_2 -IsoP not only affects vascular and bronchial smooth muscle function but also modulates cellular proliferation [57]. In addition to the above metabolites, nonenzymic oxidation of ARA produces isofurans and isoketals. Similarly, nonenzymic lipid mediators of DHA oxidation include neuroprostanes, neurofurans, and neuroketals. All these mediators are reliable indices of oxidative stress in vivo [6, 30, 52].

Ceramide and sphingomyelin are major components of lipid rafts in neural membranes. In neurodegenerative diseases, ceramide and ROS modulate intracellular ion channels, cell proliferation, and apoptotic cell death [52]. Ceramide triggers the generation of ROS and increases oxidative stress in many mammalian cells and animal models of neurodegenerative diseases [30, 38] (Fig. 7.3). Moreover, inhibition of ROS-generating enzymes or treatment of antioxidants impairs sphingomyelinase

activation and generation of ceramide. Ceramide-enriched raft platforms are important redox signaling platforms that amplify activation of ROS-generating enzymes (e.g., NADPH oxidase family enzymes) and sphingomyelinases [52].

The brain is the richest source of cholesterol in the body. Most brain cholesterol is present in myelin sheets and in cellular membranes. The presence of cholesterol in neural membranes is necessary for optimal fluidity, neural plasticity, and synaptic transmission. The structure of cholesterol makes it susceptible to a variety of radical attacks because of the 5,6-double bond and the concomitant vinylic methylene group at C-7 in the B ring. In addition, at C-17, cholesterol has an isooctyl side chain, which undergoes enzymic oxidation largely by cytochrome *P*450-dependent oxygenases, but this site of oxidation is typically not a target for ROS relevant to cellular biochemistry [60, 61]. ROS-mediated oxidation of cholesterol results in formation of three major products, cholesta-4,6-dien-3-ol, cholesta-4,6-dien-3-one, and cholesta-3,5-dien-7-one. In brain cytochrome *P*450-dependent oxygenases transform cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol. Cholesterol is also oxidized to cholesterol oxides and converted into cholesterol ester via acyl-CoA:cholesterol acyltransferase [62, 63].

Transformation of cholesterol into 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol is an important mechanism for the excretion of cholesterol from the brain. It promotes the maintenance of brain cholesterol homeostasis [64, 65]. Hydroxycholesterols produce apoptotic cell death in the brain through the activation of caspases [63, 64]. It should be noted that metabolism of phospholipid, sphingolipid, and cholesterol are closely interrelated and interconnected. For example, glycerophospholipid-derived lipid mediators (ARA and PGs) regulate sphingolipid metabolism by modulating SMase activity, and sphingolipid-derived lipid mediators (ceramide, ceramide phosphate, and sphingosine) regulate phospholipid metabolism by stimulating PLA₂ activity [6, 66] (Fig. 7.3). Moreover, many cell stimuli modulate more than one enzyme at the same time; this adds complexity to the regulation of phospholipid, sphingolipid, and cholesterol metabolism. Under physiological conditions, homeostasis among phospholipid, sphingolipid, and cholesterol metabolism and activities of PLA₂, COX, LOX, SMase, and cytochrome *P*450 oxygenases are based not only on levels of lipid mediators and organization of signaling network but also on the complexity and interconnectedness of their metabolism. In neurodegenerative diseases, elevations in PLA₂, COX, LOX, SMase, and cytochrome *P*450 oxygenases and marked alterations in levels of lipid mediators disturb the signaling networks, resulting in loss of communication among glycerophospholipid, sphingolipid, and cholesterol metabolism. This process threatens the integrity of neural cell lipid homeostasis, resulting in neural cell death [6, 51, 66, 67].

7.3 INFLAMMATION AND NEURODEGENERATIVE DISEASES

Inflammation is a protective mechanism, which not only isolates injured brain cells from uninjured cells but also destroys injured neurons and initiates the repair of the extracellular matrix [67]. Although the main mediators of neuroinflammation are microglial cells, recent studies indicate that astrocytes, neurons, and oligodendrocytes also contribute to inflammatory response. In the normal healthy brain, resting microglial cells have a ramified morphology (a small cell soma and numerous branching processes) and are associated with monitoring their microenvironment in the brain. In neurodegenerative diseases, the resting microglia are activated and transformed into activated microglia, which are characterized by amoeboid morphology. Activated microglial cells migrate rapidly to the site where the neurodegenerative process is taking place. They not only engulf dead cells but also clear cellular debris. Thus activated microglial

cells act as immunocompetent macrophage-like cells in the injured or infected brain. They not only mediate the innate defense system but also interact with cellular debris through scavenger receptors. These receptors bind to cellular debris, and microglial phagocytic receptors signal via immunoreceptor tyrosine-based activation motif-containing adaptor proteins that promote phagocytosis of extracellular material. Insufficient clearance by microglia appears to be prevalent in neurodegenerative diseases such as AD [68, 69]. Similarly, in neurodegenerative diseases astrocytes also undergo activation in the areas showing the accumulation of aggregated proteins and may release a variety of signaling molecules, such as cytokines, chemokines, and growth factors. They also show increased expression of glial fibrillary acidic protein, vimentin, and nestin [70]. Thus astrocytes provide homeostatic control of the extracellular environment of the neurons and respond to various stimuli such as disease and chemical or physical damage.

In neurodegenerative diseases, the chronic activation of microglia promotes neuronal damage through the release of glutamate, ROS, NO, proinflammatory cytokines, proteinases, and complement proteins [67, 71, 72], which can exert deleterious as well as beneficial effects on the surrounding tissue. These factors propagate and maintain neuroinflammation by a number of mechanisms, including the activation of multiple forms of PLA₂, COX, and LOX, generating PAF, and proinflammatory prostaglandins [39]. Thus at the molecular level in neurodegenerative diseases, inflammation is accompanied by the activation of isoforms of PLA₂, COX, LOX, SMases, and cholesterol hydroxylases, increase in expression of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) and chemokines, and production and accumulation of prostaglandins, leukotrienes, thromboxanes, ceramides, and hydroxyl- and ketocholesterols. Some prostaglandins and leukotrienes produce proinflammatory effects by interacting with their receptors, whereas others (prostaglandin J₂ and lipoxins) cause anti-inflammatory and antiapoptotic effects in the brain [30, 52]. Emerging evidence suggests that activated microglia and astrocytes take part in neuroinflammation, which is different from nonneural (peripheral) inflammation due to the involvement of a complex network of neural cells, signaling molecules, and lipid mediators that occur within the brain. As mentioned above, neuroinflammatory responses include microglial and astroglial cell proliferation and migration of microglia and astrocytes toward the site where aggregated proteins are accumulating and the release of cytotoxic and inflammatory mediators (cytokines and chemokines, advanced glycation end products; Fc fragment of antibodies, and other complement factors) [10, 30, 38, 66, 67, 73, 74].

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine), another potent proinflammatory mediator, is synthesized from a specific subclass of PtdCho that contains an ether bond at the *sn*-1 position of the glycerol backbone during oxidative stress [75–77]. PAF exerts its neurochemical effects by activating the PAF receptors on neural cell surfaces [75, 77]. Stimulation of PAF receptors promotes transcriptional activation of a number of genes including immediate-early genes including *c-fos*, *c-jun*, and *krox-24*, cytokines, enzymes such as cyclooxygenase-2, and growth factors [78, 79]. The activation of these genes by PAF can be blocked by the PAF antagonist BN 52021 [80]. Excessive levels of PAF have been implicated in inflammatory syndrome, epileptic seizures, bacterial meningitis, multiple sclerosis, prion diseases, Miller–Dieker lissencephaly, and HIV replication associated with AIDS dementia complex [77, 81]. PAF has also been implicated in the neuronal damage in AD. Although the mechanisms linking neural cell injury to PAF levels are fully understood, the activation of PAF receptors is accompanied by the mobilization of calcium through calcium channels and from intracellular stores and enhanced turnover of PtdCho, PlsEtn, and PtdIns [82] via the activation of phospholipases and generation of ROS through the oxidation of ARA. These ROS interact with NF- κ B/I κ B complex in the cytoplasm [30, 67, 83]. Upon stimulation I κ B is rapidly phosphorylated, ubiquitinated, and then degraded by proteasomes, resulting in the release and subsequent nuclear translocation of active NF- κ B [84]. In the nucleus, NF- κ B mediates the transcription of many genes implicated in inflammatory and immune responses (Fig. 7.3). These genes include COX-2, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, TNF- α , IL-1 β , IL-6, sPLA₂, inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs). Expression of these proteins may promote neuronal growth cone collapse and neurodegeneration in neurological disorders [77, 85].

At the molecular level, neuroinflammation includes two phases. One phase is associated with the generation of proinflammatory lipid mediators such as eicosanoids and platelet-activating factor, and the other phase is called resolution of inflammation, a turning off mechanism by which neural cells limit tissue injury [66, 86]. Resolution involves the synthesis of proresolving and anti-inflammatory eicosanoids. The molecular mechanism of resolution remains elusive [87]. However, lipoxins [88], PGD₂ and PGJ₂ [89], and docosanoids (resolvins, neuroprotectins, and maresins) [40, 41, 86] (Fig. 7.4) have been reported to play an important role during resolution. Resolvins, neuroprotectins, and lipoxins are potent anti-inflammatory and proresolving molecules that act through specific G protein-coupled

receptors, which suppress the expression of proinflammatory cytokines. In addition, studies on the effect of nitrooleic acid on cultured dorsal root ganglion (DRG) neurons indicate that this lipid mediator is present in normal and inflamed mammalian tissues at up to micromolar concentrations and exhibits anti-inflammatory signaling actions [90].

Emerging evidence suggests that neuroinflammation includes not only long-standing activation of glial cells (microglia and astrocytes) and subsequent sustained release of the above-mentioned inflammatory mediators but also elevation in oxidative and nitrosative stress [91]. The sustained release of inflammatory mediators is required for the neuroinflammatory cycle, activating additional microglia and promoting their proliferation, which promotes the further release of inflammatory factors. Sustained nature of the neuroinflammation often facilitates abnormalities in the blood-brain barrier (BBB), which increases infiltration of peripheral macrophages into the brain parenchyma to further intensify the neuroinflammation [92, 93]. The duration and intensity of neuroinflammatory response dictate whether neuroinflammation is detrimental or beneficial.

7.4 SIGNIFICANCE OF INTERPLAY AMONG EXCITOTOXICITY, OXIDATIVE STRESS, AND NEUROINFLAMMATION

It is well known that intensity of excitotoxicity, oxidative stress, and neuroinflammation are significantly increased in normal aged brain compared to adult brain [67]. The onset of many neurodegenerative diseases is associated not only with increased intensity but prolonged duration of interactions among excitotoxicity, oxidative stress, and neuroinflammation [4, 94]. Initially, the coordinated and controlled interplay among excitotoxicity, oxidative stress, and neuroinflammation in normal aged human brain may cause some abnormalities in motor and cognitive performance, but in neurodegenerative diseases an enhanced rate of interplay among excitotoxicity, oxidative stress, and neuroinflammation may turn on specific genes that affect only a specific neuronal population in a particular region where neuronal degeneration occurs (Fig. 7.6) [94, 95]. This proposal is supported by the hypothesis that the nature of neuron-neuron connections as well as interactions between neurons and glial cells is essential for determining the selective neuronal vulnerability of neurons in neurodegenerative diseases [66, 67, 96]. This interplay may be a common mechanism of brain damage in neurotraumatic diseases (stroke, spinal cord injury, and traumatic head injury) as well as neurodegenerative diseases such as AD, PD, HD, and ALS [6, 7]. In neurotraumatic diseases,

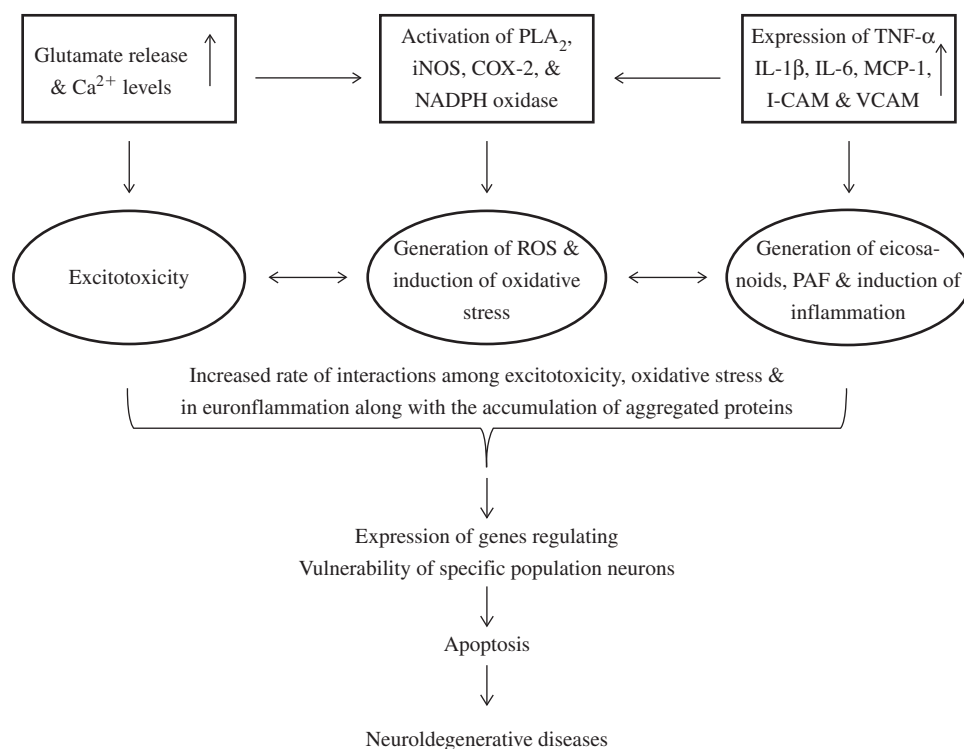


Fig. 7.6 Interactions among excitotoxicity, oxidative stress, and neuroinflammation in neurodegenerative diseases.

neurons die rapidly (within hours to days) because of sudden lack of oxygen, reduction in ATP, and sudden collapse of ionic gradient along with an acute inflammatory response that is accompanied by increased production of cytokines, chemokines, acute-phase proteins, and complement factors. In contrast, in neurodegenerative diseases some oxygen, nutrients, and ATP are available to neurons, and ionic homeostasis is maintained to a limited extent. These processes, along with consistent and continuous chronic inflammatory response and oxidative stress, result in a neurodegenerative process that takes several years to develop. Little is known about the rate of neurodegeneration and clinical expression of neurodegenerative diseases with age. As stated above, neurodegenerative diseases commence late in life and are accompanied by the loss of specific neuronal populations, synapses, and accumulation of misfolded protein aggregates [2, 4, 30]. The chemical nature of the misfolded protein aggregate is different in each neurodegenerative disease. Furthermore, each neurodegenerative disease has a separate etiology with distinct morphological and pathophysiological characteristics. However, they share similar common terminal neurochemical processes such as excitotoxicity, oxidative stress, and inflammation [6, 7]. Importantly, increased intensity and prolonged duration of interplay among excitotoxicity, oxidative stress, and neuroinflammation impair neurogenesis, a process involved in the maturation of stem cells into

new functional neurons, supporting the view that adult neurogenesis may be involved in regenerative attempts and the neuroplasticity of the nervous system [97]. In addition in neurodegenerative diseases, it is proposed that neurons increase their defenses by developing compensatory responses (oxidative strength) [98, 99] aimed to avoid or at least reduce cellular damage caused by the interplay among excitotoxicity, oxidative stress, and neuroinflammation. This hypothesis is supported by studies on A β deposition in AD. It is stated that A β may not be the initiator of AD pathogenesis, but rather a downstream protective adaptation mechanism developed by cells in response to coordinated and upregulated interplay among excitotoxicity, oxidative stress, and neuroinflammation [98–100]. A proposal on the neuroprotective role of A β explains why many aged individuals, despite having a high number of senile plaques in their brain, show little or no alteration in cognitive function. Accumulating evidence suggests that more studies are required on neurochemical aspects of neurodegenerative diseases in patients with neurodegenerative diseases.

7.5 CONCLUSION

ROS are generally generated in normal physiological conditions at low levels and are scavenged by endogenous antioxidants, such as superoxide dismutase,

glutathione peroxidase, catalase and small molecules such as vitamin C and E. Oxidative stress refers to the pathological states in which increased ROS production exceeds the antioxidant capacity of brain tissue to neutralize ROS. Although low levels of ROS are needed for normal neural cell function, high ROS levels damage neuronal plasma membrane and membranes of subcellular organelles directly (e.g., through peroxidation). ROS react with metals, nitrogen, or carbon to form intermediates that react with proteins (e.g., through nitration and nitrosylation). Oxidative stress also damages DNA or RNA, including mtDNA. Neuroinflammation is a neuroprotective process, in which the brain responds to infections, diseases, and injuries. Microglial cells play a major role in inducing and maintaining the intensity and duration of neuroinflammation through the release of glutamate, ROS, proinflammatory cytokines, and NO. Inhibition of microglial activation leads to the amelioration of neurodegeneration. It is becoming increasingly evident that increase in intensity and duration of interactions among excitotoxicity, oxidative stress and neuroinflammation, alterations in calcium homeostasis, mitochondrial and proteasomal dysfunction, and ROS-mediated protein aggregation play a crucial role in the development and progression of many neurodegenerative diseases.

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NEUROSTEROIDS IN OXIDATIVE STRESS-MEDIATED INJURY IN ALZHEIMER DISEASE

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8.1 INTRODUCTION

The brain is considered to be especially vulnerable to oxidative stress due to high levels of prooxidant factors and relatively low antioxidant defence. Putative prooxidant factors consist of a high metabolic rate, high levels of unsaturated fatty acids that readily undergo lipid peroxidation reactions, and relatively high levels of iron in some brain regions that facilitate hydroxyl radical formation from Fenton reactions [1]. Furthermore, neuronal activity results in high levels of intracellular calcium ions after depolarization that are linked to activation of phospholipase A₂, release of arachidonic acid, and subsequent formation of reactive oxygen species (ROS) from cyclooxygenase and lipoxygenase reactions (Fig. 8.1). Calcium ions also facilitate mitochondrial depolarization with release of mitochondrial factors that promote ROS formation. Furthermore, calcium ions are required for nitric oxide synthesis via endothelial and neuronal nitric oxide synthases (eNOS and nNOS). The brain contains relatively high levels of nitric oxide that can give rise to formation of highly reactive peroxynitrite. Also, catecholamine metabolism involves increased ROS formation: Superoxide can be generated from semiquinone formation, and hydrogen peroxide is released as a by-product of catecholamine synthesis by tyrosine hydroxylase and degradation by monoamine oxidases.

Despite these prooxidant factors, the brain possesses only relatively low levels of antioxidant defenses. Catalase activity is extremely low in brain tissue, and glutathione peroxidase as well as superoxide dismutase show low activity compared with other organs such as liver, heart, and kidney [2]. As a consequence, increased levels of ROS can be especially detrimental to brain tissue. Oxidative stress has accordingly been suggested to be a primary factor in the pathogenesis of several chronic neurodegenerative disorders, most prominently Alzheimer disease (AD), Parkinson disease (PD), and Huntington disease (HD).

Several studies, mainly in animals, suggest neurosteroid involvement in neuroprotection [3]. However in humans, the role of neurosteroidogenesis in the regulation of degenerative mechanisms is unknown. Since the process of neurosteroid biosynthesis is a pivotal mechanism intervening in the protection or viability of nerve cells, it might be regulated or significantly affected under oxidative stress conditions. However, the key factors interacting with neurosteroid biosynthesis under pathological conditions are poorly understood. New findings demonstrate an amino acid sequence-dependent action of amyloid- β (A β) on neurosteroidogenic pathways [4]. The data also indicate that, unlike progesterone neosynthesis, regulation of endogenous estradiol formation by pathogenic factors may be a deciding process controlling cell death mechanisms. Targeting estradiol

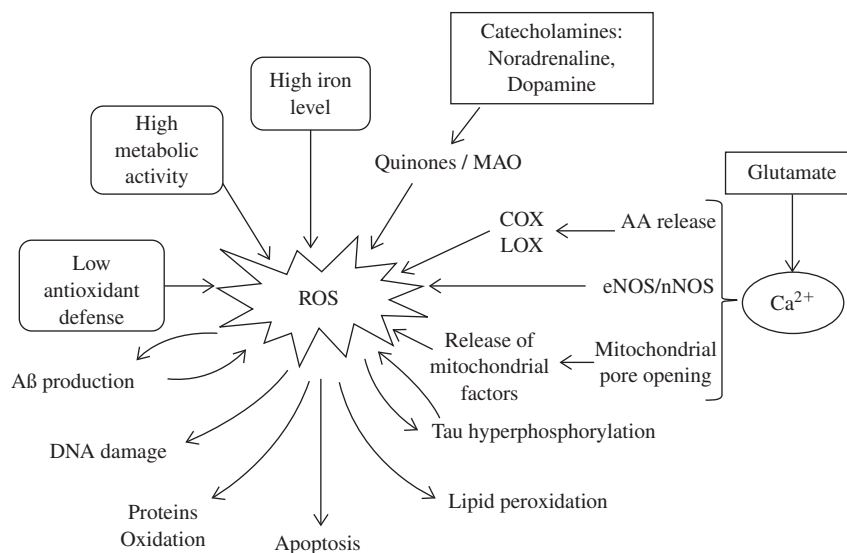


Fig. 8.1 Factors contributing to the accumulation of reactive oxygen species (ROS) in brain tissue and their implications in Alzheimer disease. See text for details. MAO, monoamine oxidase; COX, cyclooxygenase; LOX, lipoxygenase; AA, arachidonic acid; eNOS/nNOS, endothelial/neuronal nitric oxide synthase; A β , amyloid- β peptide.

biosynthetic pathways in nerve cells may therefore be interesting in development of neuroprotective strategies.

8.2 EVIDENCE FOR A PATHOLOGICAL ROLE FOR OXIDATIVE STRESS IN AD

AD is a neurodegenerative brain disease and the most common form of dementia among the elderly. It is characterized by clinical symptoms of severe and progressive loss of memory, language skills, as well as spatial and temporal orientation. The pathology of AD is characterized by extracellular senile plaques, composed of aggregated amyloid- β peptide, and intracellular neurofibrillary tangles, consisting of aggregates of abnormally hyperphosphorylated Tau protein, and is accompanied by mitochondrial dysfunction, but the mechanisms underlying AD-related dysfunction and neurodegeneration are poorly understood.

Identification of factors that contribute to the pathology of AD comes from epidemiological as well as genetic studies: AD can be classified into two different forms, rare familial forms (FAD), in which the disease onset is at an age younger than 60 years, and the vast majority of sporadic AD cases, in which onset occurs at an age over 60. Both forms of AD show the same clinical symptoms and neuropathology. Genetic studies in FAD patients have identified mutations in the genes encoding for the amyloid precursor protein (APP) and for the presenilins PS1 and PS2 that cause an autosomal inherited form of AD with 100% penetrance. These FAD mutations consistently lead to increased production of A β from

its precursor protein APP, which prompted Hardy and Higgins [5] to suggest a direct and pathological role for A β accumulation in the development of AD. In the sporadic form of the disease, several risk factors have been found that increase the risk to develop the disease but—unlike FAD mutations—do not necessarily lead to development of AD. Aging is by far the most important risk factor for AD, but the apolipoprotein E4 allele and female sex also predispose to the development of AD.

Immunohistochemical studies of postmortem AD brains have established that neurons undergo apoptotic cell death. Since ROS can elicit apoptotic signaling, the hypothesis that oxidative stress is involved in the pathogenic steps that lead to the development of AD was proposed in the 1990s by several groups [6–9]. There is a large body of evidence in support of this hypothesis: Oxidative stress has been repeatedly shown to be associated with A β toxicity and with risk factors for sporadic AD—mostly aging and the apolipoprotein E4 genotype.

8.2.1 Evidence for a Role for Oxidative Stress in Sporadic AD

Oxidative stress has been associated with the risk factors for sporadic AD, most prominently with aging [10], suggesting that an age-associated rise in accumulation of ROS can render the brain more vulnerable to the development of AD. Consequently, increased markers of oxidative stress have been found in AD patients: Several studies have reported elevated levels of lipid peroxidation products, oxidatively modified proteins, and oxidized DNA and RNA bases in brains and cerebrospinal

fluid from AD patients compared to age-matched nondemented control subjects [11–13]. Furthermore, tissue samples from AD brains display a higher susceptibility to *in vitro* oxidation [14], suggesting an impairment of antioxidant defense in AD patients. Reports on antioxidant parameters in AD brains have, however, been contradictory so far. Several antioxidant enzymes have been studied in AD brains with inconsistent results, but the majority of reports found elevations in antioxidant enzymes, suggesting an upregulation of antioxidant defense in response to increased ROS levels [15]. Interestingly, upregulation of antioxidant defence was more pronounced in female patients, and levels of 4-hydroxynonenal (HNE), a neurotoxic aldehyde derived from lipid peroxidation reactions, were elevated in female compared to male patients. These findings suggest that brains from female AD patients are under higher oxidative pressure [15], consistent with epidemiological findings that AD is more frequent in postmenopausal women compared to age-matched men. This observation can possibly be linked to the lack of sexual hormones, especially estrogens, that can modulate cognitive function and nonreproductive behaviours in humans and other mammalian species [16]. Potential sources of ROS in AD brains include ROS derived from impaired mitochondrial function [17, 18] and secondary ROS formation due to inflammatory reactions. Furthermore, increased monoamine oxidase B activity and increased levels of potentially pro-oxidative heavy metals like iron have been identified in AD brains [19, 20] and in patients with mild cognitive impairment (MCI), the “clinical precursor of AD,” suggesting that oxidative stress is an early event of the disease [21].

Apart from aging, the apolipoprotein E4 allele is the second most important risk factor for the development of AD. Apolipoprotein E seems to play a role in brain lipid metabolism and neuronal and glial development. It can exist in three different alleles, E2, E3, and E4, which differ in only two amino acids: The E2 isoform contains two and the E3 isoform one cysteine residue, while the E4 isoform contains none. Carriers of the apolipoprotein E4 are at increased risk to develop sporadic AD, especially when they are homozygous carriers. The apolipoprotein E4 allele has been associated with increased oxidative damage in AD brains, with the greatest impact in homozygotic carriers [22] and an increased susceptibility to cell death in lymphocytes from carriers bearing at least one E4 allele [23]. *In vitro* studies have evidenced that apolipoprotein E4 is less efficient in binding HNE, a cytotoxic lipid peroxidation product. These findings suggest that the Apo E4 isoform increases susceptibility to oxidative damage, thereby possibly predisposing to the development of AD.

8.2.2 Oxidative Stress and Toxicity of Mutant APP, Presenilins, and Tau

Since the proposal of the amyloid hypothesis of AD, toxic mechanisms caused by mutant APP and presenilins related to an increased production of A β have been extensively studied. Cells exposed to A β undergo apoptotic cell death, and the toxicity of A β has been shown to be related to the production of ROS [24, 25]. Furthermore, toxicity of A β depends on its aggregation state, which can be influenced by oxidation. Thus oxidative stress can cause formation of toxic A β species, which in turn can further exacerbate accumulation of ROS in a vicious cycle (Fig. 8.1). This could also explain why the prevalence of AD increases with advancing age—due to rising oxidative stress levels with aging favoring A β toxicity.

Toxicity of A β is also evident in cell cultures overexpressing APP/A β . PC12 cells transfected with mutant APP Swedish showed higher sensitivity to ROS-induced cell death and increased mitochondrial impairment after challenge with hydrogen peroxide [26]. Similar observations were obtained in human neuroblastoma cells (SH-SY5Y) overexpressing human wild-type APP (wtAPP) [27]. The study demonstrated that chronic exposure to A β protein resulted in activity changes of complexes III and IV of the oxidative phosphorylation system (OXPHOS) in mitochondria coupled with a drop of ATP levels and an increase of ROS production, which may finally instigate loss of synapses and neuronal cell death in AD. Furthermore, treatments of untransfected SH-SY5H cells with A β or human amylin aggregates induced an increase of ROS production and had a negative impact on mitochondrial respiration by their action on OXPHOS system [25].

Toxicity of A β has also been evidenced in animal models of the disease. Mice transgenic for mutant APP have high levels of A β in their brains and show an age-dependent formation of A β plaques similar to the plaques found in AD patients. Increased markers of oxidative stress have been detected in brains of transgenic mice bearing mutant APP, accompanied by markers for mitochondrial damage [28]. Furthermore, mutant APP transgenic mice show reduced levels of the antioxidant enzyme copper/zinc superoxide dismutase [29]. In agreement, increased markers of oxidative stress and reduced antioxidant defense by catalase as well as a trend toward reduced activity of SOD were found in brains from FAD patients [30]. The results provide an important link of studies on toxicity of mutant APP in cell culture and animal models mimicking the pathogenesis of the disease in FAD patients, all of them bearing mutations finally causing an increased generation of A β .

Mutations in the presenilins PS1 and PS2 account for the majority of FAD cases and have similarly been linked with oxidative stress. Oxidative toxicity of mutant

presenilins can be either (i) due to increased formation of toxic A β , especially the A β ₁₋₄₂ isoform, or (ii) due to direct toxic effects of mutant presenilins. Several mutations in the presenilins have been found that consistently lead to increased production of the long A β ₁₋₄₂ from its precursor protein APP [31], resulting in increased A β levels and toxicity via the above-mentioned mechanisms. Expression of mutant presenilins in cell culture and transgenic mice sensitizes cells to apoptotic stimuli by increasing ROS production and mitochondrial damage [32, 33]. Furthermore, brains from PS1 mutant transgenic mice display reduced activities of antioxidant enzymes [34], and lymphocytes from these mice display increased sensitivity to apoptosis accompanied by high intracellular ROS and calcium levels [35]. Interestingly, increased ROS accumulation, disturbed calcium homeostasis, and diminished levels of antioxidants have also been identified in peripheral cells from FAD patients bearing APP or PS mutations as well as in cells from sporadic AD patients [36]. These results suggest that the oxidative toxicity observed in transgenic animal models of the disease can indeed play an important role to the pathogenesis of sporadic as well as familial AD in humans.

The second main hallmark lesion of AD is intracellular neurofibrillary tangles (NFTs) built up of hyperphosphorylated Tau. This protein may block the transport of mitochondria, leading to energy deprivation and oxidative stress at the synapse as well as to neurodegeneration [37]. Functional analysis showed mitochondrial dysfunction in transgenic mice (pR5 mice) expressing P301L mutation of Tau, with a reduced complex I activity and, with age, impaired mitochondrial respiration and ATP synthesis. Mitochondrial dysfunction was associated with higher levels of ROS in aged pR5 mice. Increased Tau pathology as in aged homozygous pR5 mice revealed modified lipid peroxidation levels and upregulation of antioxidant enzymes in response to oxidative stress. These findings demonstrated for the first time that not only the A β but also the Tau pathology acts on the enzyme metabolism of the brain and the oxidative conditions in AD. However, more recently, the successful development of double, and even triple, transgenic mouse models has facilitated the investigation of pathogenic mechanisms in AD and assisted in an understanding of the interplay of A β and Tau on bioenergetics processes in vivo [37]. These findings support the idea that A β and Tau act synergistically in amplifying mitochondrial respiratory deficits, mainly of complex I and IV activities [18].

8.2.3 Is Oxidative Stress an Early Event in the Pathogenesis of AD?

From the above evidence it can be concluded that oxidative stress is a feature of sporadic as well as familial forms

of AD. However, it remains to be elucidated whether oxidative stress is a primary factor in the pathogenesis of the disease or only a secondary contributing mechanism. The fact that oxidative damage and mitochondrial dysfunction can be detected at early stages in animal models [28]—even before the presence of A β plaques [38]—and that oxidative stress parameters have been detected at highest levels in early stages of the disease in AD patients [39] suggest that oxidative stress is a primary event in the course of the disease. This is supported by studies that reported a reduced risk of AD in users of antioxidant vitamin supplements [40]. Although further clinical trials are needed, antioxidant therapeutic approaches seem to be most effective at very early stages of AD and are even better utilized to modulate disease risk.

8.3 NEUROSTEROIDS

Steroid hormones are now well-defined molecules that are mainly produced by endocrine glands, such as adrenal gland, gonads, and placenta. They are involved in the control of a lot of physiological processes, from reproductive behavior to stress responsiveness. With their ability to cross cellular membranes, and thus the blood-brain barrier, steroid hormones have also an important role in the development, maturation, and differentiation of the central and peripheral nervous systems.

Three decades ago, Baulieu and co-workers were the first to show a steroid production within the nervous system itself. They discovered that some steroids, such as pregnenolone (PREG) and dehydroepiandrosterone (DHEA), were more concentrated in the brain than in the plasma [41]. In addition, they could show that the level of these steroids remained elevated in the brain even after adrenalectomy and castration. These molecules are now called “neurosteroids” and are defined as neuroactive steroids that are synthesized within the nervous system, independently of peripheral endocrine glands. Enzymatic activities of proteins involved in steroidogenesis have been shown in many regions of the central and peripheral nervous systems, in neurons as well as in glial cells [42]. Pharmacological and behavioral studies showed that neurosteroids were implicated in several physiological mechanisms, for example, cognition, anxiety, depression, neuroprotection, and even nociception [43]. Thus the conservation of the ability to produce neurosteroids during vertebrates’ evolution suggests that this category of molecules is important for living beings.

8.3.1 Biosynthesis of Neurosteroids

Neurosteroids derive from cholesterol and other blood-borne steroidal precursors. The first step of neurosteroidogenesis is the transfer of molecules of cholesterol from

the outer to the inner mitochondrial membrane. Free cholesterol accumulates outside of mitochondria and binds to the steroidogenic acute regulatory protein (StAR), a hormone-induced mitochondria-targeted protein that initiates cholesterol transfer into mitochondria. Then, molecules are transported inside mitochondria by a protein complex including translocator protein (TSPO), a cholesterol-binding mitochondrial protein also known under the name of peripheral-type benzodiazepine receptor (PTBR), which permits cholesterol transfer into mitochondria and subsequent steroid formation [44]. This translocation from the outer membrane to the inner membrane of mitochondria is the rate-limiting step in the production of neurosteroids. In fact, the ability of cholesterol to enter into mitochondria to be available to cytochrome *P450* cholesterol side chain cleavage enzyme (*P450_{scc}*), located in the inner side of the mitochondrial membrane and responsible for the conversion of cholesterol to PREG, will determine the efficiency of steroidogenesis.

PREG, precursor of all steroid hormones, is then transported to the endoplasmic reticulum, where it is metabolized to form neuroactive steroids (Fig. 8.2). The next enzymatic step in neurosteroidogenesis is the conversion of PREG into DHEA by the cytochrome *P450_{c17}* enzyme (*P450_{c17}*), also called 17 α -hydroxylase/17,20 lyase. This enzyme catalyzes the 17 α -hydroxylation of PREG in a two-step reaction that gives first 17-hydroxyPREG (17OH-PREG) and then the final product, DHEA. Each step requires the molecules NADPH and O₂.

PREG can also be catalyzed by another enzyme called 3 β -hydroxysteroid dehydrogenase (3 β -HSD) into progesterone (PROG). In general, 3 β -HSD uses NAD⁺ as a cofactor to oxidize hydroxysteroids, such as PREG, 17OH-PREG, and DHEA, into their respective ketosteroids, PROG, 17OH-PROG, and androstenedione. Then, neurosteroidogenesis follows two main pathways with PROG as precursor: the androgen/estrogen pathway and the corticoid pathway.

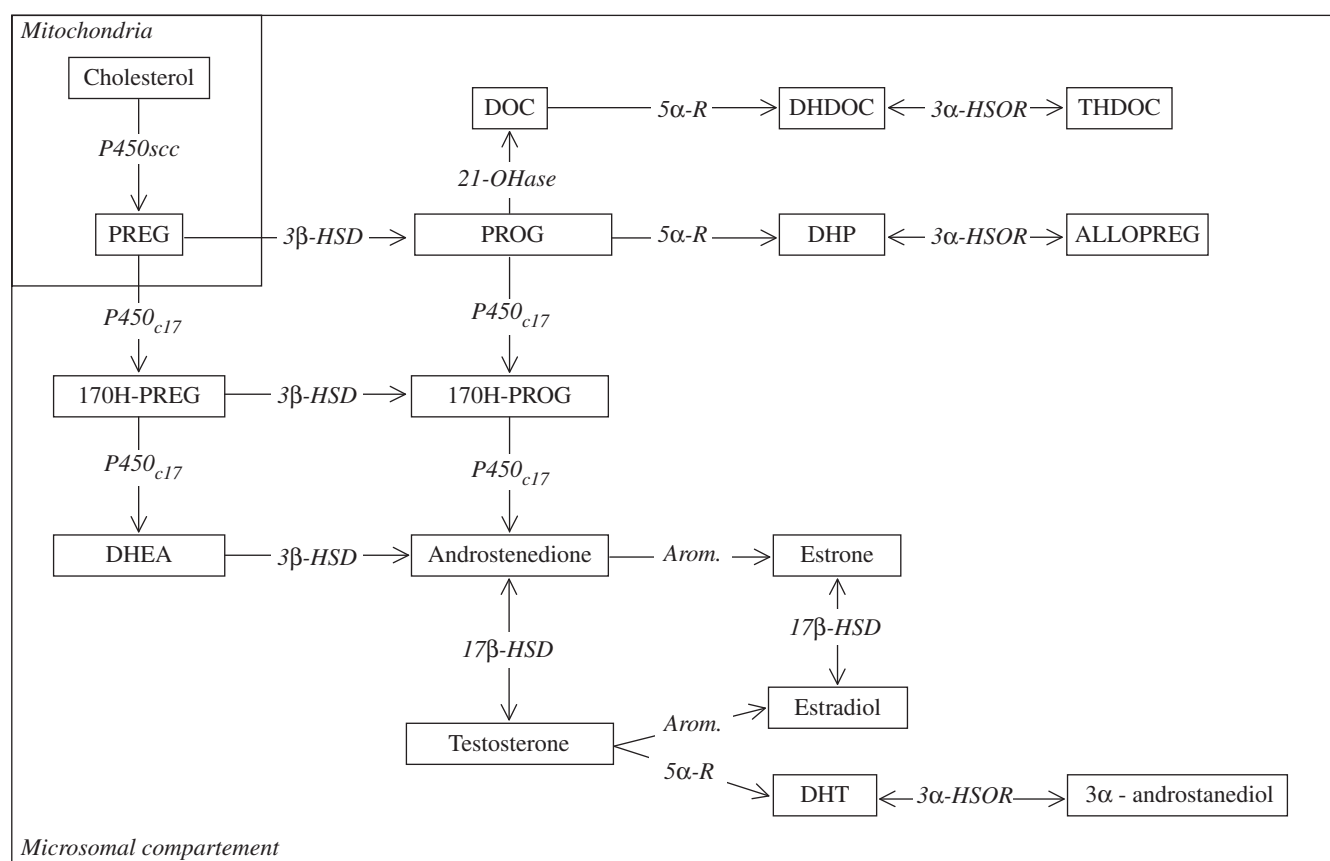


Fig. 8.2 Main biochemical pathways for neurosteroid biosynthesis and metabolism in the vertebrate brain. 17OH-PREG, 17-hydroxypregnenolone; 17OH-PROG, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone; DHDOC, dihydroxydeoxycorticosterone; THDOC, tetrahydroxydeoxycorticosterone; DHP, dihydroprogesterone; ALLOPREG, allopregnenolone; DHT, dihydrotestosterone; *P450_{scc}*, cytochrome *P450* cholesterol side chain cleavage; *P450_{c17}*, cytochrome *P450_{c17}*; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase; 5 α -R, 5 α -reductase; Arom., aromatase; 21-OHase, 21-hydroxylase; 3 α -HSOR, 3 α -hydroxysteroid oxydoreductase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase.

In the first pathway, PROG is metabolized by the same enzyme as PREG, the cytochrome *P450c17*, which converts PROG into androstenedione with the 17-hydroxyPROG as an intermediate product of reaction. Androstenedione is then converted in a reversible manner into testosterone by another hydroxysteroid dehydrogenase called 17 β -HSD. Of note, this enzyme possesses several isoforms, and one of them, 17 β -HSD-10, also called ABAD (A β binding alcohol dehydrogenase) or ERAB (endoplasmic reticulum-associated amyloid β -peptide binding protein), is in mitochondrial matrix. This isoform was recently linked to AD because of its ability to bind A β peptide, thus inducing mitochondrial dysfunction [45]. 17 β -HSD is also responsible for the reversible conversion of estrone, an estrogen stemming from aromatization of androstenedione by the enzyme aromatase into estradiol. The second way to synthesize estrogens is via testosterone molecules, which can, in turn, be metabolized into estradiol by aromatase or continued metabolism via the androgen pathway. The 5 α -reductase enzyme (5 α -R), a microsomal NADPH-dependent protein, intervenes at this level and catalyzes the transfer of two atoms of hydrogen from NADH to form the 5 α -reduced metabolite of testosterone, dihydrotestosterone (DHT) [42]. Finally, the enzyme 3 α -hydroxysteroid oxido-reductase (3 α -HSOR), also called 3 α -hydroxysteroid dehydrogenase, catalyzes the reversible conversion of DHT into the neuroactive steroid 3 α -androstenediol.

The latter enzymes also intervene at another level, in the second main steroidogenic pathway which starts with PROG. In fact, PROG is successively metabolized by the 5 α -R and the 3 α -HSOR to form dihydroprogestosterone (DHP) and 3 α /5 α -tetrahydroprogestosterone (3 α /5 α -THP), also known under the name allopregnenolone, another neuroactive steroid.

To finish by the corticoid pathway, molecules of deoxycorticosterone (DOC), stemming from the transformation of PROG by the enzyme 21-hydroxylase (21-OHase), are in turn successively converted into dihydroxydeoxycorticosterone (DHDOC) and tetrahydroxydeoxycorticosterone (THDOC) by the 5 α -R and the 3 α -HSOR, respectively.

8.3.2 Mechanisms of Action of Neurosteroids

The main role of steroid hormones produced by gonads or adrenal glands is now well defined and consists of a feedback loop on the hypothalamus-pituitary axis, to inhibit or activate their own synthesis. Thus they act at a distance from their glands of origin in an endocrine way. In contrast, neurosteroids are synthesized by the nervous system and act on the nervous system in an autocrine/paracrine configuration [46]. The ability of neurosteroids

to cross cellular membranes allows them to act on nuclear receptors and to have a genomic action by regulating gene transcription. This action seems to be important during neonatal life, when it has been shown that neurosteroids, such as PROG or estradiol, are able to promote dendritic growth, spinogenesis, synaptogenesis, and cell survival, particularly in the cerebellum [47]. The most studied steroid nuclear receptors are the estrogen receptors α and β , which are expressed in metabolic tissue such as adipose tissue, skeletal muscle, liver, and pancreas, as well as in the central nervous system. Some studies have demonstrated that these receptors play a role in the regulation of glucose homeostasis and lipid metabolism [48], whereas other studies showed that they were also implicated in neuroprotection [49].

Neurosteroids can also act via membrane receptors and play a role in general as allosteric modulators of neurotransmitter receptors. For example, sulfate esters of DHEA and PREG are known to be excitatory neurosteroids and can inhibit the effect of GABA, an inhibitor neurotransmitter, at physiological concentration by acting via the GABA $_A$ receptor [46]. On the contrary, allopregnenolone is a positive allosteric modulator of GABA $_A$ receptors, strengthening the effects of GABA. PREG sulfate can also potentiate the effect of the main excitatory neurotransmitter glutamate by binding to *N*-methyl-D-aspartate (NMDA) receptors. On the other hand, it is well known that neurosteroids modulate neurotransmitter binding sites or receptors including calcium channels and P2X receptors in the brain, spinal cord, as well as dorsal root ganglia (DRG) [50].

Furthermore, recent clinical and pharmaceutical studies showed that estrogens can interact with several neurotransmitter systems, such as the cholinergic and serotonergic systems, to influence cognitive performance in animals and humans [51]. Thus neurosteroids seem to play an important role in the nervous system during development as well as in adult brain, by regulating gene transcription and different neurotransmitter systems. Their implication was already demonstrated in several pathologies, such as AD or neuropathic pain [42, 52]. Thus it can be speculated that they might be an important therapeutic target to develop in the next years.

8.4 NEUROSTEROIDS AND OXIDATIVE STRESS

During recent years, a growing body of evidence has shown that neurosteroids, in particular estrogens, are implicated in the regulation of oxidative stress by acting on mitochondria [53]. However, on one hand, depending

on the level of oxidative stress within cells estrogens can have a protective effect or, on the contrary, show a negative action on cell survival. On the other hand, oxidative stress itself can have an effect on neurosteroid production within nerve cells.

8.4.1 Regulation of Neurosteroidogenesis by Oxidative Stress and A β Peptide

It is established that steroids can be synthesized by nonglandular tissue within the nervous system. But the regulation of their biosynthesis is still poorly understood. Recent findings showed that several glial cells, in particular oligodendrocytes, upregulated their production of

DHEA under oxidative stress conditions induced by treatment with A β peptide or Fe²⁺ [54]. Modulation of neurosteroid production was also observed in neuroblastoma (SH-SY5Y) cells overexpressing the key AD proteins, APP/A β or Tau (Fig. 8.3) [52]. Indeed, overexpression of human wild-type Tau (hTau 40) protein induced an increase in production of progesterone, 3 α -androstenediol, and 17-hydroxyprogesterone, in contrast to overexpression of the abnormally hyperphosphorylated Tau bearing the P301L mutation and leading to a decrease in the production of these neurosteroids. In parallel, a decrease of progesterone and 17-hydroxyprogesterone production was observed in cells expressing human wild-type APP (wtAPP), whereas

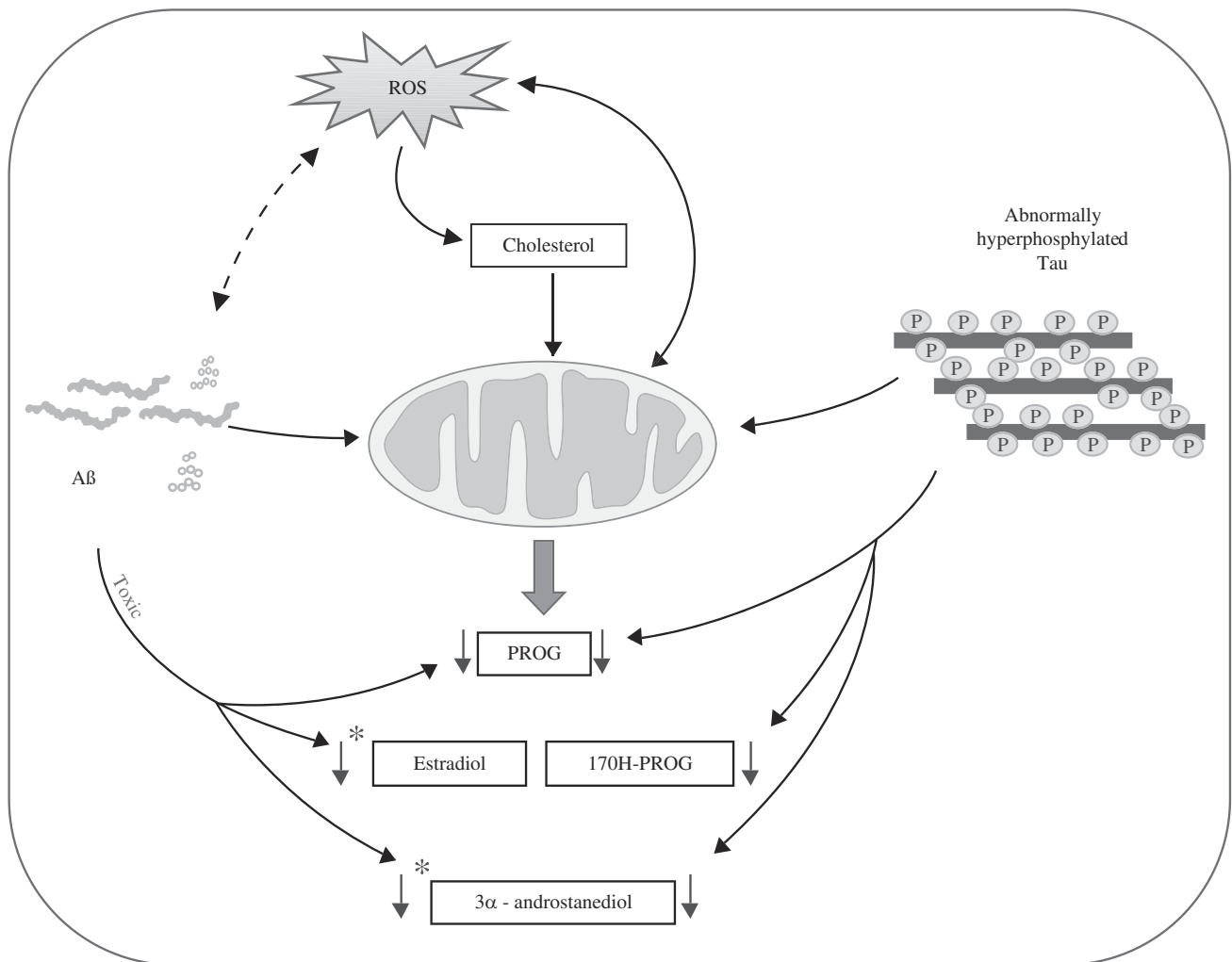


Fig. 8.3 Effect of toxic concentrations of A β peptides and abnormally hyperphosphorylated Tau protein on neurosteroid biosynthesis. A β induced a drop of the level of progesterone (PROG), estradiol, and 3 α -androstenediol by acting on reactive oxygen species (ROS) formation and mitochondrial function and/or directly on steroidogenesis. The presence of abnormally hyperphosphorylated Tau protein had the same effect by inducing a decrease of progesterone, 17-hydroxyprogesterone (17OH-PROG), and 3 α -androstenediol. On the other hand, it has been shown that nontoxic concentrations of A β induced an increase in estradiol and 3 α -androstenediol levels (this pathway is marked by *). (*See color insert.*)

3 α -androstenediol and estradiol level were increased. The latter finding was additionally confirmed with in vitro treatment experiments [4]. APPwt SH-SY5Y cells secrete A β levels within the nanomolar concentration range. Consistently, treatment of native SH-SY5Y cells with “nontoxic,” that is, non-cell death-inducing, A β_{1-42} concentrations in vitro revealed an increase in estradiol production, whereas toxic A β_{1-42} concentrations within the micromolar range strongly reduced estradiol levels revealing the exact opposite effect. Of note, oxidative stress was able to modify neogenesis of neurosteroids in a similar pattern [55]. In fact, treatment with H₂O₂ for 24 h or 48 h induced a decrease of estradiol synthesis that was correlated to a downregulation of the aromatase, the enzyme responsible for estradiol formation from testosterone. Furthermore, an increase of cell death was observed in the presence of letrozole, an inhibitor of aromatase. This suggests that endogenous estradiol formation is very important for human neuroblastoma cells and plays a critical role in cell survival. Interestingly, when cells were pretreated with estradiol, it was possible to rescue neuroblastoma cells from H₂O₂ as well as from letrozole-evoked death. In agreement, similar results were also found in stress condition experiments using heavy metals, such as cobalt and mercury, and once again estradiol was able to reverse their deleterious effect by reducing oxidative stress and β -amyloid secretion [56].

8.4.2 Estrogens and Neuroprotection

Neuroprotective effects of estrogens against a variety of brain injuries have been described for many years. Treatment with 17 β -estradiol was able to protect the brain against excitotoxicity, A β peptide-induced toxicity, free radical generators, and ischemia in animal studies [53], but the basis of these effects is still poorly understood. It was recognized from former studies that estrogen depletion in postmenopausal women represents a significant risk factor for the development of AD and that an estrogen replacement therapy may decrease this risk and even delay disease progression [57, 58].

However, results from the “Woman’s Health Initiative Memory Study” (WHIMS) including 4532 postmenopausal women aged over 68 years indicated a twofold increase in dementia after 4.2 years of treatment (p.o. treatment with premarin plus medroxyprogesterone). In addition, it indicated potential risks for breast cancer, pulmonary embolism, and stroke [59, 60]. Besides warrantable criticism with regard to the synthetic hormones used in the WHIMS trial, the outcome results were unexpected and disappointing. One can ask the question, “How could it be that so many scientific studies before the WHIMS trial were wrong?” Thus the currently prevailing view points about the “critical window

hypothesis” [16] are asking about the critical period in which one might expect a neuroprotective effect to occur. The results of the WHIMS study also initiated a discussion about a two-edged effect of estradiol. Thus estradiol can possibly also exhibit a “prooxidant effect” in the presence of ongoing oxidative stress [53]. Thereby, estradiol can be hydroxylated to give catecholestrogens that can enter a redox cycle generating superoxide radical. In an oxidative environment, this redox cycling can lead to a continuous formation of ROS that amplifies even more oxidative stress and increases neuronal loss.

On the contrary, animal studies, especially in rodents and transgenic mice models for AD, seem to confirm positive effects of estrogen treatment on the pathophysiology of the disease. It has been shown that treatment with estrogen in mice expressing mutations in human APP (Swedish and Indiana) had an impact on APP processing, decreasing levels of A β and so its aggregation into plaques [61]. In triple transgenic AD mice, depletion of sex steroid hormones induced by ovariectomy in adult females significantly increased A β accumulation and had a negative impact on cognitive performance [62]. Treatment of these ovariectomized mice with estrogens was able to prevent these effects. Of note, when PROG was administered in combination with estrogens, the beneficial effects on A β accumulation were blocked but not effects on cognitive performance. Furthermore, PROG reduced Tau hyperphosphorylation when administered alone. This suggests that estrogen and PROG can interact to regulate APP processing but can also act independently on different AD pathways.

At the cellular level, estrogen was able to activate antioxidant defense systems by reducing ROS production, limiting mitochondrial protein and DNA damage, and improving the activity of the electron chain transport during oxidative phosphorylation [53]. Thus estrogen can have direct antioxidant effects by increasing reduced glutathione (GSH) levels and decreasing oxidative DNA damage in mitochondria of ovariectomized female rats [63]. This is correlated with an upregulation of the expression of two enzymes: manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase, both of them implicated in the antioxidant defense system. Of note, estrogen can modulate the redox state of cells by intervening in several signaling pathways, such as MAPK, G protein-regulated signaling, NF- κ B, c-fos, CREB, phosphatidylinositol-3-kinase, PKC, and Ca²⁺ influx [3, 64]. On the basis of this complex mode of action, estrogen seems to be able to decrease oxidative stress markers, including lipid peroxidation, protein oxidation, and DNA damage.

Recently, it has been proposed that estrogens exert their beneficial effects by acting directly on mitochondria

via estrogen receptor β (ER β) [65]. In fact, incubation of isolated mitochondria from rat brain with estradiol leads to a decrease of H₂O₂ production by this organelle coupled with an increase of the mitochondrial membrane potential. Moreover, estradiol seems to prevent the release of cytochrome *c* by mitochondria (a mechanism known to induce apoptosis of cells by activating the caspase cascade in the cytoplasm), which increases the efficiency of the respiratory chain. In addition, estrogens are able to bind to nuclear receptors, such as estrogen receptor α and β (ER α/β), and to act as transcription factors. Thus estrogens enhanced the expression of the antiapoptotic proteins, Bcl-2 and Bcl-xL, preventing the initialization of the cell death program by mitochondria [3]. They were also able to increase the expression of F1 subunits of ATP synthase and glucose transporter subunits and regulate enzymes involved in the tricarboxylic acid (TCA) cycle, which has the effect of improving glucose utilization by cells.

As described recently, estrogens can have an effect on the transcription of mitochondrial genes, especially on the electron transport chain components [66]. Treatment of ovariectomized female rats with estradiol induced an increase of mitochondrial respiratory function translated into an enhancement of O₂ consumption and coupled to an increased expression and activity of cytochrome *c* oxidase (electron transport chain complex IV).

Finally, another means for estrogens to avoid negative effects of oxidative stress is to regulate calcium homeostasis by inducing mitochondrial sequestration of cytosolic calcium [53]. In fact, an imbalance of calcium handling can lead to an increase of ROS production by activating the enzyme nitric oxide synthase, which can sensitize neural cells to oxidative damage. It has been shown that estradiol treatment of primary hippocampal neurons was able to potentiate glutamatergic response via NMDA receptor, which resulted in an increased influx of calcium in cells. This effect was coupled with an induction of mitochondrial sequestration of cytosolic calcium and an increase of the mitochondrial calcium load tolerability, to avoid calcium-induced excitotoxicity and to promote cell survival.

8.5 CONCLUSION

In summary, it is now clear that oxidative stress is an important actor involved in AD pathophysiology and intervenes already at an early disease stage. Furthermore, good evidence is provided that neurosteroids, such as estrogens, are able to limit oxidative damage by reducing lipid peroxidation, protein oxidation, Ca²⁺ overload in cytosol, and DNA damage in mitochondria as well as in the nucleus. These effects are mediated by

several mechanisms, from transcription of genes coding for antioxidant enzymes to the regulation of antiapoptotic pathways, by way of improvement of mitochondrial respiratory chain efficiency and glucose metabolism. Thus, with their abilities to counter excess oxidative stress, estrogens seem to be able to prevent AD-related toxic mechanisms, such as A β peptide aggregation, Tau hyperphosphorylation, and neuronal loss. Better human studies taking into account the critical window hypothesis are essential before drawing a final conclusion on efficacy of neurosteroids in prevention of AD.

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OXIDATIVE STRESS IN ADULT NEUROGENESIS AND IN THE PATHOGENESIS OF ALZHEIMER DISEASE

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9.1 INTRODUCTION

Alzheimer disease (AD) is the most common form of dementia among the elderly. It is a neurodegenerative disease that affects more than 26 million patients worldwide and for which there is still no cure [1, 2]. AD is characterized by memory and cognitive deficits, amyloid deposits, neurofibrillary tangles, neurodegeneration, aneuploidy, and genome damage [3]. Genetic mutations and genetic, acquired, and environmental risk factors, particularly neuroinflammation and oxidative stress, are the main causes of AD [4]. New research in adult neurogenesis and neural stem cells (NSCs) suggests that newly generated neuronal cells of the adult brain, particularly of the hippocampus, may be involved in AD and may be affected by reactive oxygen species (ROS) and oxidative stress [5, 6].

ROS are free radical reactive substances formed by the incomplete reduction of oxygen. They include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^{\bullet}) and peroxynitrite anions ($ONOO^-$). Free radicals, such as ROS, are produced physiologically by cells and are involved in cell signaling and metabolism [7]. They are also damaging and toxic to the cells. They oxidize cytoplasmic and membrane proteins, lipids, and nucleic acids. Oxidation of proteins and nucleic acids and peroxidation of lipids compromise cellular functions. Cells are protected against the toxicity of ROS by enzymes, such as superoxide dismutase, catalase, and glutathione. These enzymes act as natural antioxidants.

Under physiological conditions, the balance between the generation and degradation of ROS by cells and organisms is highly regulated. Oxidative stress occurs when the level of free radicals exceeds the antioxidant capacity of the cells [8]. It results from an elevation of production of free radicals, from a decrease in the scavenging of free radicals, or from a decrease in the mechanisms used to repair oxidized macromolecules. Oxidative stress and the excessive production of ROS are deleterious conditions leading to cellular dysfunction and cell death, via apoptosis or necrosis [9]. Oxidative stress plays a key role in the development of numerous pathologies, particularly neurodegenerative diseases such as AD [9]. In this chapter we review and discuss the involvement and contribution of oxidative stress and ROS to adult neurogenesis and to the pathogenesis of AD.

9.2 ALZHEIMER DISEASE

AD is a neurodegenerative disease characterized by memory and cognitive deficits and anosmia [10–12]. The disease is initially associated with the loss of nerve cells in areas of the brain that are vital to memory and cognition, such as the entorhinal cortex, hippocampus, and neocortex. As the disease progresses, other regions of the brain are affected, leading to severe incapacity and death, [12]. AD is characterized in the brain by the presence of amyloid plaques and neurofibrillary tangles, the histopathological hallmarks of the disease, and

aneuploidy [13]. AD is the most common form of dementia among the elderly. The disease affects 30% of individuals over the age of 80 [14]. Age is the principal risk factor for AD. The incidence of the disease doubles every 5 years after age 65 [14]. AD affects more than 35 million patients worldwide, a number expected to quadruple by 2050 as the population ages [15]. Late-onset AD (LOAD) refers to cases of AD diagnosed after the age of 65. Early-onset AD (EOAD) refers to cases of AD diagnosed before age 65. LOAD accounts for the vast majority, over 93%, of all cases of AD.

9.2.1 Inherited and Sporadic Forms of Alzheimer Disease

The inherited form of AD, also known as familial Alzheimer disease (FAD), is caused by mutations in so-called familial Alzheimer genes, such as the gene of β -amyloid precursor protein (APP), the presenilin-1 (*PSEN-1*) gene, and the presenilin-2 (*PSEN-2*) gene [16]. It is a rare form of the disease, affecting about 200 families in the world. The sporadic form of AD is caused by a combination of genetic, acquired, and environmental risk factors [17]. These include the presence of the ApoE varepsilon 4 allele (*ApoE4*), the presence of variants in at least two different clusters of intronic sequences in the neuronal sortilin-related receptor (*SORL1*) gene, hypertension, diabetes, neuroinflammation, and oxidative stress [18–21]. The sporadic form of AD is the most common form of the disease. It accounts for most cases of LOAD, whereas the inherited form of AD accounts for most cases of EOAD.

9.2.2 Amyloid Plaques and Neurofibrillary Tangles

Amyloid plaques and neurofibrillary tangles are deposits of proteins distributed throughout the brain of patients with AD, particularly in the regions of degeneration such as the entorhinal cortex, hippocampus, and temporal, frontal, and inferior parietal lobes [22]. Amyloid plaques are primarily composed of extracellular aggregates of protein β -amyloid or amyloid fibrils and of the serine protease inhibitor α 1-antichymotrypsin [22]. Protein β -amyloid is a 42-amino acid peptide that is derived from the posttranscriptional maturation of APP [23]. The abnormal processing of APP results in the aggregation of protein amyloid and the formation of amyloid plaques. Neurofibrillary tangles are intracellular aggregates of hyperphosphorylated Tau protein. Tau protein is a microtubule-associated phosphoprotein [24]. The hyperphosphorylation of Tau protein by kinases results in the aggregation of Tau protein and the breakdown of microtubules that are involved in cell structure, intracellular transport, and cell division [25]. Amyloid plaques

and neurofibrillary tangles are two of the probable causes of the pathogenesis of AD; amyloid plaques and neurofibrillary tangles that would cause cell death in the brain [26]. In support of this contention, rat embryonic cortical neurons cultured with toxic concentrations of protein β -amyloid reenter the cell cycle and die by apoptosis in vitro [27].

9.2.3 Aneuploidy and Expression of Proteins of the Cell Cycle

Aneuploidy is a landmark of AD pathology. Preparations of lymphocytes from patients with AD, EOAD and LOAD, reveal an increase of aneuploidy in cells, particularly for chromosomes 13, 18, and 21 [28, 29]. Since the cells that are the most likely to develop aneuploidy are dividing cells, the nondisjunction of chromosomes during cell division in stem cells or somatic cells that retain their ability to divide is at the origin of aneuploidy in lymphocytes of patients with AD [30]. A substantial number of neurons, 4% to 10%, in regions of degeneration, such as the hippocampus, of the brain of AD patients express proteins of the cell cycle and are aneuploid. Among the proteins of the cell cycle expressed by nerve cells in regions of degeneration are the proliferating nuclear antigen, Ki-67, cyclin D, cyclin-dependent kinase 4, and cyclin B1 [31, 32]. Nerve cells are postmitotic. The forced expression of oncogenes in postmitotic nerve cells causes cell death rather than cell proliferation. Hence, nerve cells in regions of degeneration that express proteins of the cell cycle or are aneuploid would originate from abortive cell cycle reentry, leading to apoptosis, or cycle reentry and gene duplication, without cell division, leading to aneuploidy [33, 34].

The deregulation and/or reexpression of proteins controlling the cell cycle and aneuploidy in nerve cells would underlie the neurodegenerative process and pathogenesis of AD. In aneuploid cells, the genetic imbalance results in the overexpression of genes by the cells. This has tremendous consequences for the development of AD, as genes involved in the pathology of AD would further contribute to the pathogenesis of the disease, as a result of their overexpression. The genes of ApoE, APP, PSEN-1, PSEN-2 and TAU are located on chromosomes 19, 21, 1, 14, and 17, respectively [35–38]. Aneuploidy for chromosomes 19, 21, 1, 14, and/or 17 would result in the overexpression of ApoE and in an increased risk for individuals who have *ApoE4* in their genetic make-up of developing the sporadic form of AD, would result in the overexpression of APP and promote the formation of amyloid plaques, would promote the formation of amyloid plaques in patients carrying FAD mutations on PSEN genes and contribute to the pathogenesis of EOAD, and/or would result in the overexpression of Tau

TABLE 9.1 Processes Associated with Neurogenesis and Oxidative Stress in Alzheimer Disease

DNA damage	Mutational events, modified base 8-hydroxydeoxyguanosine, strand breaks, and large deletions
Cell cycle	Activity in controlling the cell cycle, cell cycle reentry of nerve cells, aneuploidy, particularly for chromosome 17 carrying the <i>TAU</i> gene
Protein oxidation	Enzymatic and mitogenic pathways, such as EGF and VEGF pathways, the stress-activated protein kinases JNK and p38, JAK/STAT, protein kinase C pathways, and histone deacetylase

Oxidative stress is a risk factor for developing Alzheimer disease (AD). Proteins, lipids, and nucleic acids elicit high rate of oxidation in patients with AD. The nuclear and mitochondrial DNA in degenerated regions of the brain of patients with AD elicit lesions. Reactive oxygen species (ROS) and oxidative stress promote cell cycle reentry of nerve cells and aneuploidy, particularly for chromosome 17 carrying the *TAU* gene. Aneuploidy for chromosome 17 in newly generated cells of the adult brain would promote the expression of Tau proteins in the hippocampus and neurodegeneration. ROS and oxidative stress contribute to the pathogenesis of AD by promoting cell death and neurodegeneration.

protein and promote the formation of neurofibrillary tangles. Cells of AD patients elicit an elevation of aneuploidy, particularly for chromosomes 13, 18, and 21 [28, 29, 39]. Hence, aneuploid cells in AD patients are likely to contribute to the pathogenesis of the disease with high probability as a result of the overexpression of genes involved in AD. It is proposed that the genetic imbalance in aneuploid nerve cells signifies that they are fated to die [33]. Their relatively high percentage at any one time in regions of degeneration in AD brains suggests that they will undergo a slow death process, unlike apoptosis. These cells may live in this state for months, possibly up to 1 year [33]. Hence, the deregulation and/or reexpression of proteins controlling the cell cycle in nerve cells would underlie the neurodegenerative process in AD.

9.2.4 DNA Damage

The nuclear and mitochondrial DNA in degenerated regions of the brain of patients with AD elicit lesions and mutational events, such as DNA oxidation, as evidenced by the presence of the modified base 8-hydroxydeoxyguanosine, strand breaks, and large deletions (Table 9.1) [40]. Proteins, lipids, and nucleic acids elicit a high rate of oxidation in patients with AD, particularly in regions containing amyloid plaques and neurofibrillary tangles in the brain [41]. AD is a neurodegenerative disease caused by an excessive rate of damage in the genome [42].

9.3 ADULT NEUROGENESIS AND ENHANCED NEUROGENESIS IN ALZHEIMER DISEASE

9.3.1 Adult Neurogenesis and Neural Stem Cells in Mammals

Neurogenesis occurs primarily in two regions of the adult mammalian brain, the dentate gyrus (DG) of the hippocampus and the anterior region of the subventricular zone (SVZ), in various species including in humans [43–46]. Newly generated neuronal cells in the

subgranular zone of the DG migrate to the granule cell layer, where they differentiate into granulelike cells and extend axonal projections to the CA3 region of the Ammon's horn [47, 48]. Newly generated neuronal cells in the SVZ migrate through the rostro-migratory stream to the olfactory bulb, where they differentiate into granule and periglomerular interneurons [49, 50]. Newly generated neuronal cells in the DG establish synaptic contacts and functional connections with neighboring and target cells [48, 51–53]. They establish mossy fiber-like synapses with target cells of the CA3 region of the Ammon's horn [53]. The number of neuronal cells generated in the adult mammalian brain is relatively low. In the DG, it is estimated that 9000 neuronal cells, corresponding to about 0.1% of the granule cell population, are generated per day in young adult mice [54]. In higher primates, such as humans and monkeys, the number of neuronal cells generated per day is lower than in rodents. In adult macaque monkey, an estimated 0.004% of the granule cell population is generated per day in the hippocampus [55]. Newly generated neuronal cells in the adult brain survive for an extended period of time, at least 2 years in the human hippocampus [43]. Newly generated neuronal cells of the adult brain may replace nerve cells born during development. They may be involved in the physiology, pathology, and plasticity of the nervous system, particularly of the hippocampus and olfactory bulb.

Newly generated neuronal cells in the adult brain would originate from a pool of residual NSCs; the self-renewing multipotent cells that generate, through a transient amplifying population of cells, the main cell types of the nervous system, nerve cells, astrocytes, and oligodendrocytes [56, 57]. In support of this contention, self-renewing multipotent NSCs have been isolated and characterized in vitro from various regions of the adult brain of mammals, including from the hippocampus and SVZ [58–60]. Neural progenitor and stem cells express molecular markers, such as the intermediate filament nestin, the transcription factors sox-2, oct-3/4, and the RNA binding protein Musashi 1 [61–64]. Hence,

neurogenesis occurs in the adult brain and NSCs reside in the adult central nervous system. This reveals that the adult brain has the potential for self-repair and may be amenable to repair. The stimulation of endogenous neural progenitor or stem cells of the adult brain and the transplantation of adult-derived neural progenitor and stem cells are proposed to repair and restore the degenerated or injured nerve pathways. To this end, the isolation and characterization of population of neural progenitor and stem cells from human biopsies and postmortem tissues provide a source of tissue for cellular therapy for the treatment of a broad range of neurological diseases and injuries, including neurodegenerative diseases such as AD and Parkinson disease, cerebral strokes, and traumatic and spinal cord injuries [65, 66].

Neurogenesis occurs constitutively in the adult brain and is modulated by a broad range of environmental stimuli, physiological and pathological conditions and processes, trophic factors/cytokines, neurotransmitters, and drugs. This includes environmental enrichment, learning and memory tasks, physical activity, AD, and epilepsy [67, 68]. The contribution of adult neurogenesis and its modulation to the physiology and pathology of the nervous system remains to be determined.

9.3.2 Neurogenesis in Patients with Alzheimer Disease and in Animal Models of Alzheimer Disease

The expression of markers of immature neuronal cells, such as doublecortin and polysialylated nerve cell adhesion molecule, is increased in hippocampal regions, particularly the DG, of the brain of patients with clinical diagnosis of AD [69]. Neurogenesis is enhanced in the DG of adult transgenic mice that express the Swedish and Indiana APP mutations, a mutant form of human APP [70]. It is reduced in the DG of PDAPP adult transgenic mice, a model of AD with age-dependent accumulation of protein β -amyloid, in the DG and SVZ of adult transgenic mice deficient for PSEN-1 and/or APP and in the DG of adult transgenic mice overexpressing variants of APP or PSEN-1 [71–75]. These results show that neurogenesis is enhanced in the hippocampus of the brain of patients with AD, but they report conflicting data and discrepancies in animal models of AD.

The apparent discrepancies in the modulation of adult neurogenesis in humans and in animal models of AD may originate from the validity of animal models as representative of AD and from limitations and pitfalls in the conducted studies. AD is a neurodegenerative disease characterized by widespread neurodegeneration and multiple and complex processes, including the deposit of amyloid, the formation of neurofibrillary tangles, and aneuploidy. Hence, transgenic mice models of AD represent models to study the genes involved in

the disease rather than the disease itself. Transgenic mice may be limited in their validity to study adult phenotype, and particularly adult neurogenesis, as the mutated genes may alter the animal's development and this may affect the adult phenotype. Aggregation of protein β -amyloid during development in APP transgenic mice may alter the development of the nervous system and therefore may have adverse consequences on adult neurogenesis [76]. The apparent discrepancies in the modulation of adult neurogenesis in human and animal models of AD as well as discrepancies in studying adult neurogenesis in human and animal models in general, may originate from the methods used for studying and quantifying cell proliferation and neurogenesis. Bromodeoxyuridine (BrdU) labeling has been the most used method to study adult neurogenesis *in vivo*. BrdU is a thymidine analog used for birthdating and monitoring cell proliferation, as BrdU integrates into the DNA of dividing cells [77]. In this paradigm, BrdU is generally administered peripherally, intraperitoneally in rodents, and the fate of the BrdU-labeled cells is followed by immunohistochemistry [78]. Multiple immunohistochemistry staining with markers of neuronal lineages combined with confocal microscopy and stereology allows qualitative and quantitative study of adult neurogenesis [79–81]. BrdU labeling as a method for studying adult neurogenesis, is not without limitations and pitfalls, particularly for the study of adult neurogenesis in AD. BrdU is not a marker for cell proliferation and neurogenesis, but a marker of DNA synthesis. As a thymidine analog, BrdU integrates into DNA of dividing cells during the S-phase of the cell cycle. Hence, studying cell proliferation and neurogenesis with the BrdU-labeling paradigm requires discriminating cell proliferation and neurogenesis of other events involving DNA synthesis, particularly abortive cell cycle reentry leading to apoptosis and cell cycle reentry and gene duplication without cell division, leading to aneuploidy [82, 83]. Since aneuploidy in the region of neurodegeneration is a landmark of AD pathology, careful analysis must be carried out when studying adult neurogenesis in animal models of AD with the BrdU-labeling paradigm. In addition, the permeability of the blood-brain barrier is affected in AD [84]. As consequence, an increase in BrdU labeling in the brain could originate from an increase in BrdU uptake rather than an increase in cell proliferation and neurogenesis. There are other considerations that may affect the analysis of BrdU-labeling studies. Among these considerations, there is no consensus on the term “neurogenesis.” Some studies only present proliferation data, whereas others present only neuronal differentiation or survival data, and in most studies, and in particular in human postmortem studies, only one time point along the pathology is analyzed. Therefore, careful

analysis and discussion of the studies must be carried out when using BrdU labeling to study adult neurogenesis, particularly in AD. Some of these limitations also apply when studying adult neurogenesis by immunohistology against markers of the cell cycle. In particular, immunohistochemistry for proteins of the cell cycle does not allow discrimination among cells reentering the cell cycle as a prelude to apoptosis, cells undergoing DNA duplication without cell division, as part of their pathological fate, and the genesis of neuronal cells [85].

Hence, these studies of adult neurogenesis in the hippocampus of the brain of patients with AD and in animal models of AD remain to be confirmed and validated. Indeed, neurogenesis might be differentially regulated along the pathogenesis. Enhanced neurogenesis in the DG of AD brain would contribute to a regenerative attempt, to compensate for the neuronal loss. It would result from damage or stimulation induction of neurogenesis and may be a consequence, rather than a cause, of the disease [86]. In the SVZ, studies show a reduction in the number of neural progenitor cells in patients with AD, as revealed by immunohistology for the markers of neural progenitor and stem cells, nestin and Musashi1 [87]. The reduction in the number of neural progenitor cells in the SVZ of AD brain may underlie the compromised olfaction associated with the disease.

9.4 OXIDATIVE STRESS: A RISK FACTOR FOR DEVELOPING ALZHEIMER DISEASE

Lipids, proteins, and nucleic acids elicit high rates of oxidation, particularly in regions of degeneration in the AD brain [41]. Oxidative stress is a risk factor for developing AD [9].

9.4.1 ROS and Oxidative Stress Promote Cell Death and Neurodegeneration

Protein β -amyloid promotes the generation of ROS in the brain of AD patients [88]. ROS, particularly generated by protein β -amyloid, and oxidative stress in the brain of patients with AD would contribute to the pathogenesis of the disease by promoting cell death and neurodegeneration. Abnormal mitotic signaling, such as abortive cell cycle reentry leading to apoptosis and cycle reentry and gene duplication, without cell division, leading to aneuploidy, underlies the process of neurodegeneration and contributes to the pathogenesis of AD [33, 34]. It is proposed that oxidative stress and abnormal mitotic signaling would both be necessary to propagate the disease. A “two-hit hypothesis” has been proposed to conciliate the activity of oxidative stress and abnormal mitotic signaling, such as abortive cell cycle

reentry and gene duplication without cell division, as causative factors of AD: Oxidative stress and abnormal mitotic signaling can act independently as initiators; however, both processes are necessary to propagate the pathogenesis of AD [89].

Abortive cell cycle reentry, cell cycle reentry and gene duplication without cell division, and DNA damage are underlying processes in the development of AD. ROS and oxidative stress promote cell cycle reentry of nerve cells and DNA damage [90]. The contribution of ROS and oxidative stress to the process of cell cycle reentry of nerve cells would support the “two-hit hypothesis” conciliating the activity of oxidative stress and abnormal mitotic signaling in the brain of AD patients. The mechanism by which ROS and oxidative stress increase the risk of developing AD, particularly through its activity on abnormal mitotic signaling, remains mostly unknown. ROS and oxidative stress would contribute to the pathogenesis of AD through their activity in controlling the cell cycle (Table 9.1). It would increase the risk of developing AD, directly or indirectly, through their oxidative activity on DNA and on various enzymatic and mitogenic pathways, such as the EGF and VEGF pathways, the stress-activated protein kinases JNK and p38, JAK/STAT, protein kinase C pathways, and histone deacetylase and through mutational events including strand breaks and large deletions (Table 9.1) [91]. Studies show that whether ROS-exposed cells undergo proliferation, growth arrest, or apoptosis depends in part on where the cell resides in the cell cycle when insulted [92]. This has implications for the mechanisms by which oxidative stress may affect neuronal cells in the adult brain, whether and how it affects mature neurons or newly generated neuronal cells in regions of neurodegeneration, such as the hippocampus. ROS and oxidative stress would also contribute to DNA damage, if the oxidation of DNA surpasses the DNA repair capacity of the cell, leading to the accumulation of mutations and the loss of genome stability, a landmark of AD pathology.

9.4.2 Oxidative Stress Promotes the Generation of Aneuploid Newly Generated Neuronal Cells in the Adult Brain

Stem cells and somatic cells that retain the ability to divide are the most likely to develop aneuploidy. Since newly generated neuronal cells of the adult brain originate from stem cells, they are most likely to develop aneuploidy. The nondisjunction of chromosomes in the process of cell division of adult neurogenesis would lead to newly generated neuronal cells that are aneuploids or to a population of aneuploid neural progenitor cells that would not proceed with their developmental program in the neurogenic regions, primarily the DG of the

hippocampus and SVZ [93]. The fate of these aneuploid newly generated neuronal cells in the adult brain remains to be elucidated. They may survive for an extended period of time or have their life span shortened, further contributing to the degenerative process in AD.

Aneuploid newly generated neuronal cells that would be aneuploid for chromosomes carrying genes involved in the pathogenesis of AD, such as the *APP*, *PSEN1* and *TAU* genes located on chromosomes 21, 14, and 17, respectively [35–38], would also further promote the development of AD, by promoting the aggregation of protein β -amyloid and Tau protein and neurodegeneration [93]. Oxidative stress promotes aneuploidy, particularly for chromosome 17 carrying the *TAU* gene (Table 9.1) [94]. Hence, ROS and oxidative stress would increase the risk of the generation of newly generated neuronal cells that are aneuploid for chromosome 17, or of a population of neural progenitor cells that are aneuploids for chromosome 17 and would not proceed with their developmental program. Aneuploidy for chromosome 17 in newly generated cells of the adult brain would promote the expression of Tau proteins in the hippocampus, and neurodegeneration. The hyperphosphorylation of Tau proteins would further promote the breakdown of microtubules and the generation of aneuploid newly generated neuronal cells [90]. Hence, oxidative stress would promote aneuploidy and the formation of neurofibrillary tangles in the neurogenic regions of the adult brain, contributing to neurodegeneration and to the pathogenesis of AD [95].

9.4.3 Antioxidants Increase Neurogenesis in the Adult Hippocampus

BrdU labeling studies show that antioxidants, such as curcumin, increase neurogenesis in the hippocampus of adult rodents [96]. This suggests that ROS and oxidative stress may decrease neurogenesis in the adult brain. On one hand, such a decrease would reduce the deleterious activity of ROS and oxidative stress in promoting the generation of aneuploid newly generated adult neuronal cells in the adult brain. On the other hand, it would limit the regenerative potential of adult neurogenesis, particularly in the hippocampus of the brain of AD patients. ROS and oxidative stress would elicit a dual activity on newly generated neuronal cells of the adult brain and on the pathogenesis of AD, promoting aneuploidy and decreasing neurogenesis [95].

9.4.4 Oxidative Stress in Patients with Mild Cognitive Impairment

The level of DNA damage and oxidized DNA bases (pyrimidines and purines) is increased by twofold in

leukocytes of patients with mild cognitive impairment and AD, compared to individuals not diagnosed with the diseases [97, 98]. Patients with mild cognitive impairment have a probability to develop AD of 12% per year or 50% within 4 years [99]. Hence, oxidative damage occurs at the early stages of AD, and oxidative stress is a risk factor and contributes to the pathogenesis of the disease [100, 101].

9.5 CONCLUSION

Oxidative stress is an environmental risk factor for developing AD, and oxidative damage occurs at the early stages of AD. Oxidative stress and ROS promote cell death and neurodegeneration and the generation of aneuploid newly generated neuronal cells in the adult brain. Adult neurogenesis is enhanced in the DG of brain of patients with AD, but ROS would reduce neurogenesis in the hippocampus. Reduced neurogenesis by ROS would limit the regenerative capacity of the adult brain and the risk of generating aneuploid newly generated neuronal cells. Further studies are mandated to elucidate the contribution of adult neurogenesis to the pathology and pathogenesis of AD. Further studies are also mandated to confirm and validate the modulation of adult neurogenesis in the brain of patients with AD, and the activity of oxidative stress and ROS on newly generated neuronal cells of the adult brain. Antioxidants have been proposed and considered for the treatment of AD, potentially delaying the development of the disease. These drugs may reduce the deleterious activities of ROS and oxidative stress in the adult brain, and in particular may reduce the generation of aneuploid newly generated neuronal cells. They may also promote the regenerative capacity of the adult brain, particularly in patients with AD.

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OXIDATIVE STRESS AND PARKINSON DISEASE

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10.1 INTRODUCTION

Parkinson disease (PD) is the most common neurodegenerative movement disorder, currently affecting more than 4 million predominantly elderly individuals worldwide [1]. Originally described as “the shaking palsy” in 1817 by the British physician James Parkinson, the disease is attended by a constellation of motoric deficits including bradykinesia (slowness in movements), postural instability, rigidity, and tremor that ultimately result in near total immobility. Although pathological changes are distributed in the PD brain [2], the principal neuropathology that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNpc), which results in a severe depletion of striatal dopamine (DA) and thereby an impaired nigrostriatal system that otherwise allows an individual to execute proper, coordinated movements. This specific pattern of neurodegeneration in PD is often accompanied by the presence of eosinophilic intracytoplasmic inclusions known as Lewy bodies (LBs) in surviving neurons in the SN as well as in other affected brain areas [2]. Accordingly, pharmacological replacement of brain DA via L-DOPA administration represents an effective symptomatic recourse for the PD patient (especially during the initial stages of the disease) and remains a clinical gold standard treatment for PD. However, neither L-DOPA nor any currently available therapies

can slow or stop the insidious degenerative process in the PD brain. Furthermore, the major drawbacks with current therapies are the inevitable loss of effectiveness and increasing drug-induced side effects as the disease progresses. Invariably, the debilitating nature and morbidity of the disease present significant social, emotional, and economic problems. As the world population rapidly ages, these problems undoubtedly will also increase.

Despite nearly two centuries of research, the etiology of PD remains elusive. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic and postmortem analysis, as well as in vitro and in vivo modeling, have contributed significantly to our understanding of the pathogenesis of the disease. In particular, the recent identification and functional characterization of several genes, including *α-synuclein*, *parkin*, *DJ-1*, *PINK1*, and *LRRK2*, whose mutations are causative of rare familial forms of PD have provided tremendous insights into the molecular pathways underlying dopaminergic neurodegeneration [3, 4]. Collectively, these studies implicate aberrant mitochondrial and protein homeostasis as key contributors to the development of PD, with oxidative stress likely acting as an important nexus between the two pathogenic events.

Notably, the brain is often thought to be particularly susceptible to oxidation-induced damage because of its high metabolic rate and its relatively reduced capacity to replenish its postmitotic neuronal populations compared with other organs. Moreover, the brain contains high

levels of phospholipids and polyunsaturated free fatty acids, both of which are prone to modifications by oxidants. For SN dopaminergic neurons, the vulnerability toward oxidative stress is further enhanced by the abundance of redox-active iron in this region of the brain, as well as by the presence of DA, whose oxidation products are potentially cytotoxic [5, 6]. Although attractive, the hypothesis that oxidative stress plays a central role in PD pathogenesis continues to be keenly debated, particularly in view of recent findings in the clinic that antioxidant strategies have failed to produce convincing protection in PD patients. Furthermore, a major question that remains unresolved to this point is whether oxidative stress represents a cause or a consequence of neurodegeneration. Here, we review and discuss both historical evidence and current thoughts about the relationship between the oxidative stress and PD, which include our own views on the topic. For the benefit of the reader, we have also included a section on DA chemistry and oxidation that summarizes the oxidative pathways for catecholamines in the genesis of cytotoxic quinones.

10.2 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

It is perhaps paradoxical to note that the production of reactive oxygen species (ROS), often regarded as the cellular “bad guys,” is an inevitable consequence of aerobic (oxygen dependent) respiration—a process needed to keep all cells in our body, including post-mitotic neurons, alive. During aerobic respiration, high-energy products (NADH and FADH₂) generated in the mitochondria via the Krebs cycle donate electrons to a series of electron carriers (complexes I–IV) on the electron transport chain (ETC) to produce a proton gradient across the inner mitochondrial membrane that is utilized by ATP synthase to drive oxidative phosphorylation of ADP to ATP (Fig. 10.1). Although molecular oxygen is typically reduced by electrons from Krebs cycle intermediates flowing through the ETC to produce H₂O, partial reduction of molecular oxygen to superoxide anion (O₂^{•−}) occurs when electrons leak from the ETC (particularly at complex I). This free radical can be converted to the highly reactive hydroxyl radical (OH[•]) via an iron-catalyzed reaction known as the Fenton reaction, or to peroxynitrite (ONOO[−]) upon reaction with nitric oxide (NO). Both hydroxyl radical and peroxynitrite are potent oxidants that can cause marked cellular damage by reacting with proteins, lipids, and nucleic acids. Further, these reactive species may also target the ETC, which results in a feedforward cycle of increasing oxidative stress and injury. It is noteworthy to

mention that NO, which is generated by nitric oxide synthase (NOS), can itself contribute to oxidative damage through *S*-nitrosylation, a reaction whereby the cysteine residues of protein are modified to nitrosothiols, often resulting in altered protein function [7]. (The role of *S*-nitrosylation and nitrosative stress in PD has been covered extensively in several excellent recent reviews [8] and is only discussed briefly in this chapter.)

To protect against potential ROS-induced damage, aerobically respiring cells have developed over the course of evolution an effective antioxidant defense mechanism that consists of a plethora of antioxidant enzymes [such as superoxide dismutase (SOD), glutathione peroxidase, and catalase] as well as nonprotein antioxidants (such as glutathione, α -tocopherol, and ascorbic acid) to keep the levels of cellular free radicals in check. For example, ROS by-products produced by mitochondrial respiration are rapidly detoxified by SOD2 (which converts superoxide to hydrogen peroxide) so that their basal levels are low and nontoxic. As glutathione (GSH) is a major antioxidant in cells, the ratio between the reduced and oxidized form of glutathione (i.e., GSH/GSSG), is often taken as an indicator of cellular redox status [9]. When a state of imbalance between the production of ROS and their clearance by the antioxidant defense system occurs, oxidative stress ensues. However, it is important to highlight that although the overproduction of ROS leading to oxidative stress is usually bad, ROS may be beneficial to cells in some instances. For example, ROS generated by phagocytes during inflammation helps to kill invading pathogens [10]. Furthermore, by virtue of the reversibility of oxidation and reduction, ROS are also used as secondary messengers in redox-based intracellular signaling in response to internal and external cues. This includes neuronal signaling processes that influence synaptic neurotransmission, synaptic plasticity, and long-term potentiation [11, 12]. Likewise, the role of NO as a signaling molecule in the brain is well characterized [13]. Thus ROS [or reactive nitrogen species (RNS)] may act as signaling or stress molecules depending on their cellular levels.

10.3 OXIDATIVE STRESS AND PD

There is certainly ample support from postmortem studies to suggest that the redox state in the PD brain is in disequilibrium. For example, several groups have reported that markers for lipid peroxidation (including 4-hydroxynonenal and malondialdehyde), protein carbonyl modifications, and even DNA and RNA oxidation are markedly elevated in the SN of postmortem PD brains [14–17], and that these ROS-induced events are

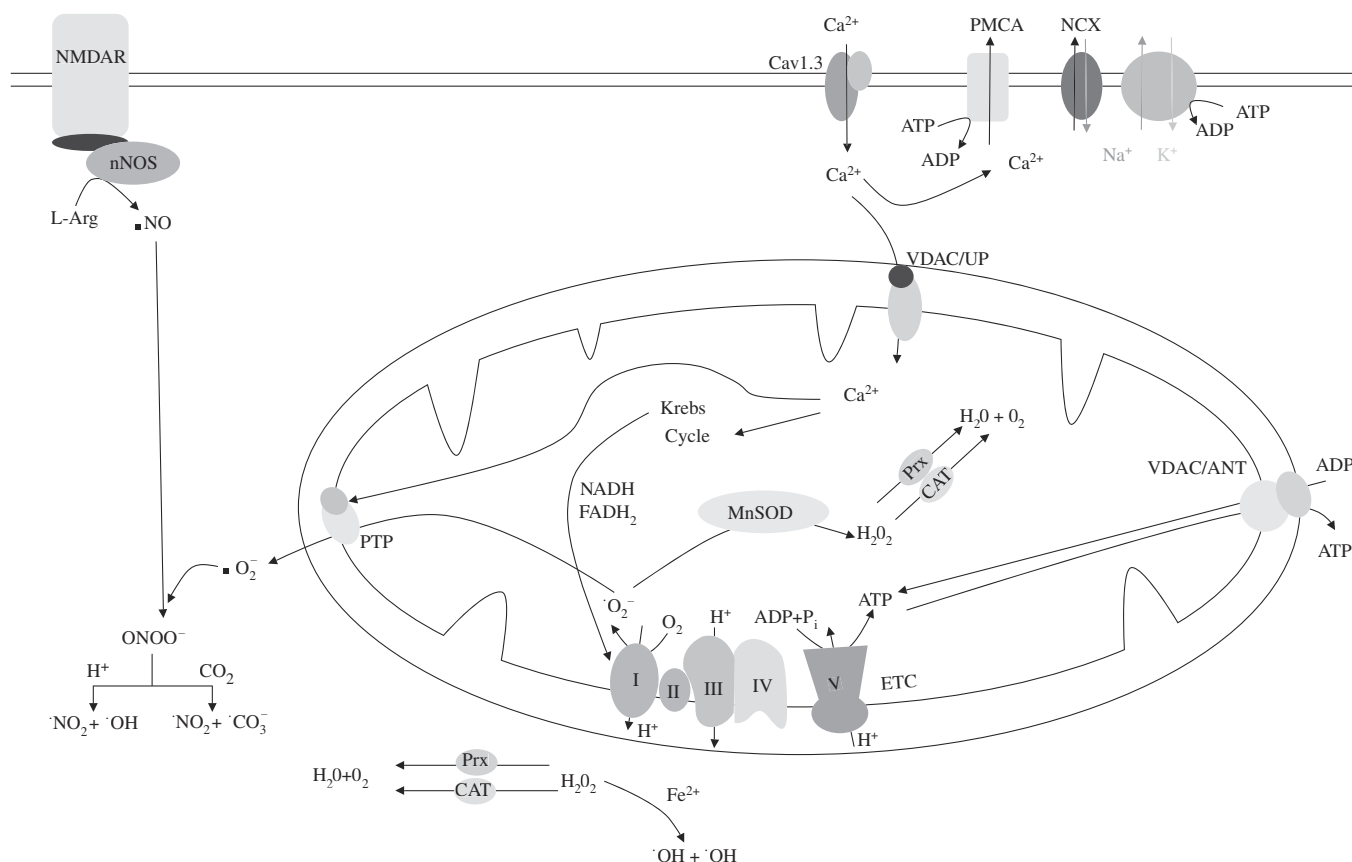


Fig. 10.1 Genesis of reactive oxygen and nitrogen species. NADH and FADH₂ produced via the Krebs cycle donate electrons to a series of electron carriers on the electron transport chain (ETC) to produce a proton gradient needed to drive the oxidative phosphorylation of ADP to ATP by ATP synthase. Leakage of electrons from the ETC (especially from complex I) occurs, leading to production of superoxide ($\text{O}_2^{\bullet-}$), which is typically detoxified into hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). H_2O_2 is then converted into H_2O and oxygen by catalase (CAT) and peroxiredoxin (Prx). H_2O_2 can also be converted to highly reactive hydroxyl radical (OH^\bullet) via the iron-catalyzed Fenton reaction. Activation of nitric oxide synthase (nNOS) as a result of *N*-methyl-D-aspartate receptor (NMDAR) over-stimulation leads to the production of nitric oxide (NO) that can react with $\text{O}_2^{\bullet-}$ to form the reactive peroxynitrite (ONOO^-). ONOO^- can react with H^+ or CO_2 in the cytosol to generate nitrogen dioxide (NO_2) and highly reactive OH^\bullet or $\text{CO}_3^{\bullet-}$. Dendritic influx of Ca^{2+} , which apparently occurs via Cav1.3 Ca^{2+} channels in SN dopaminergic neurons during pace-making, can be taken up by the mitochondria via voltage-dependent anion channels (VDAC) and Ca^{2+} uniporter (UP). Ca^{2+} entering the mitochondrial matrix can stimulate enzymes of the Krebs cycle and thereby oxidative phosphorylation and concomitantly $\text{O}_2^{\bullet-}$ production. The Ca^{2+} that entered through Cav1.3 channels is transported back across the plasma membrane at the expense of cellular energy through either the Ca^{2+} -ATPase (PMCA) or through a $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) that relies upon the Na^+ gradient maintained by the Na/K-ATPase.

accompanied by a dramatic depletion of reduced glutathione (presumably leading to a considerably weakened antioxidant defense system) [18]. Interestingly, the magnitude of GSH depletion appears to correlate well with disease progression, suggesting that GSH depletion may be taken as an (early) indicator of nigral degeneration. Changes in iron disposition also occur in the PD brain [19]. Notably, total iron is increased while ferritin is decreased in the diseased state, a condition that is expected to promote the availability of free iron capable of catalyzing the formation of ROS via the Fenton reaction. Correlating with the rise in iron content is an elevated expression of the

divalent metal transporter 1 (DMT1) in the SN dopaminergic neurons of PD patients compared to age-matched control subjects [20]. DMT1 is a major transport protein responsible for the uptake of iron and other divalent metal ions into cells, and its expression has also been reported to increase with age [21]. Finally, these studies also revealed a significant reduction in the activity of mitochondrial complex I as well as ubiquinone (coenzyme Q_{10}) in the SN of PD brains [22–24].

The relevance of the above findings derived from postmortem diseased tissues has been supported by several toxin-induced models of PD. Indeed, parkinsonian

neurotoxins such as MPTP, paraquat, and rotenone that selectively destroy nigral dopaminergic neurons in experimental models are often inhibitors of mitochondrial complex I function, the impairment of which would enhance superoxide production and thereby the formation of highly reactive free radicals that can initiate neuronal death [25]. Importantly, in these models, there is apparently a temporal and causal relationship between oxidative damage and degeneration in nigral dopaminergic neurons [26, 27]. Interestingly, MPTP-induced dopaminergic neurodegeneration and associated motor deficits in treated animals can be mitigated by iron chelation either genetically via transgenic expression of ferritin or pharmacologically via administration of clioquinol [28]. Consistent with this, newborn mice administered iron display progressive midbrain neurodegeneration that is paralleled by an increase in markers of oxidative damage as they age [10]. Together, these studies suggest that iron accumulation is a key accelerator of oxidative stress that is relevant to dopaminergic cell loss.

Recent results generated from genetic models also support a role for oxidative stress in PD pathogenesis [25]. For example, several studies have linked oxidative stress to the aggregation of α -synuclein, a presynaptic protein that is also a major component of LBs. Consistent with this, LBs are enriched with nitrated α -synuclein [29], and the protein is similarly modified in MPTP-treated mice [30]. Importantly, whereas α -synuclein overexpression in mice leads to nigral mitochondrial abnormalities [31], its genetic ablation reduces ROS production in these animals and renders them markedly resistant to degeneration when challenged by MPTP and other mitochondrial toxins [32, 33]. These results suggest that the PD-associated protein may induce oxidative stress by promoting mitochondrial dysfunction. Related to this, several studies have also demonstrated the ability of iron-mediated oxidative stress to promote α -synuclein aggregation [34–36]. Separately, we and others have shown that parkin, a ubiquitin ligase associated with recessive parkinsonism that normally functions as a potent neuroprotectant, is also susceptible to oxidative modifications [37, 38]. After its modification by a wide spectrum of oxidative stress-inducing agents such as MPTP, paraquat, and iron, the protein exhibits altered solubility properties and thereby impaired protective function [37, 38]. It is now clear that parkin function is also linked to mitochondrial homeostasis, as is the case with another recessive parkinsonism-associated protein, PINK1, suggesting that the loss of parkin or PINK1 function can precipitate oxidative stress [39]. This notion is certainly well supported by numerous studies performed in different experimental models, including mouse models [40, 41]. Interestingly, parkin also helps to regulate oxidation level in DA-containing cells by

limiting the expression of monoamine oxidases (MAO)-mitochondrial enzymes responsible for the oxidative deamination of DA (see next section), apparently by promoting the degradation of estrogen-related receptors (ERR), orphan nuclear receptors that play critical roles in the transcription regulation of many nuclear-encoded mitochondrial proteins [42, 43]. Perhaps the most direct genetic evidence supporting the role of oxidative stress in PD pathogenesis is the finding that mutations in the redox-sensitive protein DJ-1 cause an early-onset form of PD. DJ-1 is thought to operate as an antioxidant protein [44, 45], although a more recent study has classified DJ-1 as an atypical peroxiredoxin-like peroxidase that functions to scavenge mitochondrial H_2O_2 through oxidation of its cysteine at position 106 (a residue previously demonstrated by others via mutagenesis and structural analyses to be modified by oxidative stress) [45]. Notably, a pool of DJ-1 is known to be localized to the mitochondria [45, 46], and increased levels of H_2O_2 in mitochondria can be isolated from DJ-1 knockout mice [47]. Accordingly, the absence of DJ-1 may predispose dopaminergic neurons to oxidative stress-induced degeneration. Indeed, DJ-1-deficient animals are hypersensitive to pharmacological inducers of oxidative stress [48–53]. Taken together, the evidence from genetic studies supporting a relationship between oxidative stress and PD is rather compelling. However, a caveat is that none of these PD-associated genes exhibits a selective pattern of expression that could explain the vulnerability of SN dopaminergic neurons toward oxidation-mediated injury.

Why SN dopaminergic neurons are predisposed to degeneration in PD remains unclear, although the preferential accumulation of iron in this region of the brain may contribute to their vulnerability to endogenous and/or exogenous stress. Another obvious factor is DA itself. The sections below discuss the cytotoxic potential of DA reactions and address whether SN dopaminergic neurons are uniquely susceptible to degeneration by virtue of the neurotransmitter they carry.

10.3.1 DA Chemistry and Oxidation

There are two distinct pathways by which reactive metabolites can be generated from DA in the cell. The first pathway is driven by the MAO enzymes (located on the outer mitochondrial membrane) and aldehyde dehydrogenase where DA is converted to DOPAC (or dihydrophenylacetic acid) with the generation of H_2O_2 in the process. DOPAC can undergo further oxidation to form DOPAC quinones and ROS. Normally, DOPAC quinones will be conjugated to GSH. Those that fail to do so may undergo conversion to 5-S-cys-DOPAC, which can be further oxidized to other reactive species. The second

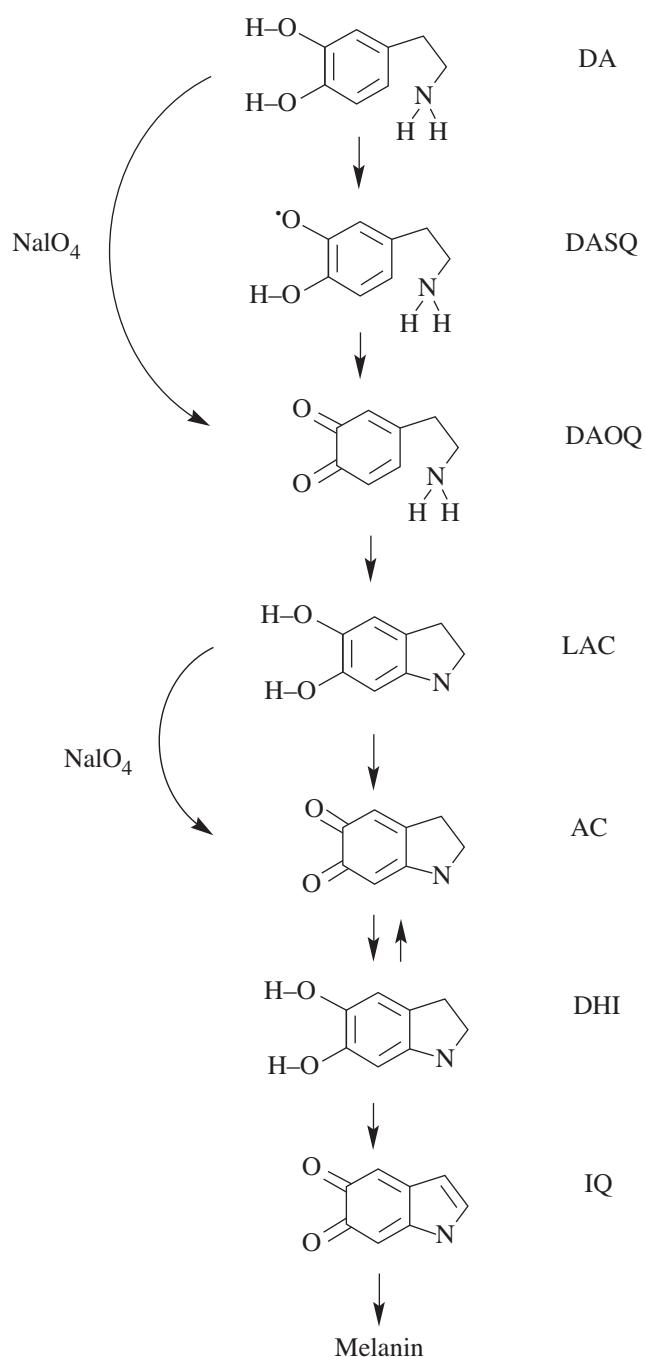


Fig. 10.2 Oxidative pathway for DA in the genesis of cytotoxic quinones. DA, dopamine; DASQ, dopamine semiquinone; DAOQ, dopamine orthoquinone; LAC, leukoaminochrome; AC, aminochrome; DHI, dihydroxyindole; IQ, indole quinone.

pathway, which is described in detail below, involves the oxidation of the catechol ring of DA to form ROS and the electron-deficient quinone (Fig. 10.2).

It has been long appreciated that the neuromelanin that accumulates in the lysosomes of catecholaminergic

neurons is the result of DA oxidation [54]. That this accumulation begins early in life and is progressive has been interpreted to mean that the process of oxidation of catecholamines to their quinone oxidation products is continuous throughout one's lifetime. Unlike the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by the tyrosinase of melanosomes in melanocytes, the oxidation of catecholamines in neurons is not tyrosinase dependent and is termed "autooxidation," a process initiated by ROS as well as by molecular oxygen with transition metal catalysis. (A recent report demonstrating the presence of tyrosinase in the brain [55] and thereby supporting a role for tyrosinase in the oxidation of catecholamines should be interpreted with caution, as the observation may be a result of contamination by leptomeningeal melanocytes.) The autooxidation of catecholamines is in addition to their oxidation by MAO. The process of autooxidation increases with increased pH and is countered by reducing agents, such as ascorbic acid. Thus the synaptic vesicle is an acidic and reducing environment where catecholamines are stable, whereas the higher pH of the cytosol enhances autooxidation [56].

In 1978 Graham described for the first time in complete form the autooxidative pathways for the catecholamines to the quinone species that polymerize to form neuromelanin [5]. As illustrated in Figure 10.2, the one-electron oxidation by O_2 can convert DA to its semiquinone radical (DASQ), which can be further oxidized to DA orthoquinone (DAOQ), reactions that generate ROS in addition. The electron-withdrawing carbonyls of DAOQ render the quinone ring electron-deficient and thus a potent electrophile that can react with external nucleophiles, such as the sulfhydryl functions of proteins and reduced glutathione. The amino function of DAOQ can then attack the quinone ring at the 6-position, an irreversible reaction that reduces the carbonyls to hydroxyl functions and yields leukoaminochrome (LAC). Once formed, LAC is then also vulnerable to autooxidation to yield the cyclized quinone aminochrome (AC). AC can then undergo tautomerization (an intramolecular rearrangement that is neither oxidation nor reduction) to 5,6-dihydroxyindole (DHI), and DHI in turn can undergo autooxidation to indole quinone (IQ) (Fig. 10.2). In parallel to the formation of eumelanin in melanosomes, it is likely that multiple species in this reaction sequence participate in the polymerization reactions that result in neuromelanin.

As one would expect, other catecholamines are also vulnerable to autooxidation to their corresponding quinone species. However, DA is much more vulnerable to autooxidation than either norepinephrine (NE) or epinephrine (E) [5]. Furthermore, the orthoquinone

products of NE and E cyclize, respectively, to leuko-noradrenochrome and leukoadrenochrome much faster than the cyclization of DAOQ to LAC. The net result of these two observations is that while DA, NE, and E can all contribute to the neuromelanin polymer as the result of autooxidation, DA stands out with its greater potential for cytotoxicity, through both generation of ROS and its oxidation to DAOQ, with its greater availability for reaction with external nucleophiles. It should be appreciated that DAOQ, and especially the orthoquinones derived from NE and E, are transient species that cannot be observed during autooxidation, as the oxidations to the orthoquinones occur more slowly than the subsequent cyclization and oxidation steps. The only methods by which the orthoquinones can be observed are electrochemical oxidation [57] and through oxidation with periodate, as first described by Graham and Jeffs [58]. Indeed, the latter paper and Graham (1978) [5] established that catechols, like 1,2-glycols, form cyclic iodate intermediates that are then hydrolyzed to the orthoquinone. The use of periodate allows specific, rapid oxidation of catecholamines that is not confounded by additional products seen with other chemical oxidants [5, 59]. It is noteworthy to mention that orthoquinones have λ_{\max} at 390 nm [5], not at 450 nm as recently reported [60], as this is the λ_{\max} of AC at high pH through the dissociation of a proton (the λ_{\max} of AC at neutral pH is 480 nm).

10.3.2 DA Oxidation and Neurotoxicity

Under normal conditions, DA is sequestered safely within synaptic vesicle. However, vesicular DA storage can be disrupted by various cellular stressors, resulting in increased cytosolic DA that can become toxic. The cytotoxicity of DA thought to play a role in neurodegeneration in PD is a product of ROS and reactive quinones generated during oxidation. Being electron deficient, DA quinones readily seek out cellular nucleophiles such as the cysteine residues on proteins. Quinone reactivity can thus deplete cellular stores of reduced glutathione. Indeed, elevated levels of 5-S-cys-DA and 5-S-cys-DOPAC are observed in the SN of PD brains [61]. Quinone reactivity can also result in the adduction and cross-linking of proteins, thereby altering their normal functions. For example, the modification of the PD-linked ubiquitin ligase parkin by the quinones derived from DA oxidation (DAQ) inactivates its enzymatic activity and promotes its aggregation [62]. Consistent with this, an increase in catecholamine-modified parkin was observed in the SNpc of postmortem PD brains [62]. Furthermore, parkin appears to be uniquely susceptible to DA-induced modifications compared to several related E3 members such as HHARI, Cbl, and

CHIP [38, 62, 63]. Given that functional parkin plays an important neuroprotective role in the brain, its modification by DAQ provides a mechanism for its dysfunction that is relevant to the pathogenesis of sporadic PD. Notably, a recent proteomic study performed by the Hastings laboratory demonstrated that DJ-1 can also be modified by DAQ [64], although it is not known whether DJ-1 function is compromised as a result. In some cases, DA conjugates can be directly toxic. For example, modification of α -synuclein by DAQ stabilizes the PD-associated protein in its protofibrillar form that can permeabilize synaptic DA-containing vesicles [65]. The resulting vesicular DA leakage, in turn, would augment the pathogenic process. Moreover, DAQ-synuclein adducts can seed the formation of oligomeric complexes that not only inhibit the normal degradation of the protein by chaperone-mediated autophagy (CMA) but also block the degradation of other CMA substrates in the process [66]. The net result is the accumulation of α -synuclein (and other proteins) and an impaired CMA function. Interestingly, α -synuclein can also interact with DA transporters and facilitate their clustering at the cell surface, the consequence of which is an acceleration of DA uptake leading to increased ROS and sensitivity toward DA-induced apoptosis [67]. Finally, several groups have shown that DA oxidation products can promote mitochondrial dysfunction through the impairment of the activity of ETC, as well as inducing mitochondrial permeability transition pore (mPTP) opening leading to organelle swelling [68–71]. Irreversible opening of the mPTP is known to promote the release of proapoptotic factors into the cytosol.

Supporting a toxic role for DA, mice administered with exogenous DA via intrastriatal injection exhibit degeneration of SN dopaminergic neurons that begins in the terminal fields and progresses to eventual loss of cell bodies, the extent of which appears to correlate with the levels of quinone-modified proteins and can be reduced by antioxidant treatment [72, 73]. Furthermore, mice engineered to express a mere 5% of normal VMAT2, a transporter that normally sequesters cytosolic DA into vesicles, display progressive nigrostriatal dopamine dysfunction that ultimately results in neurodegeneration [74]. Alongside this, elevated cysteinyl adducts to L-DOPA and DOPAC are seen early and are followed by increased striatal protein carbonyl, 3-nitrotyrosine formation, and the accumulation of α -synuclein [74]. More recently, an elegant study in mice performed by Chen and colleagues demonstrated that striatal neurons engineered to take up extracellular DA exhibit degeneration within weeks, a phenotype that is accompanied by substantial oxidative protein modifications and decrease in glutathione level [56]. That forced uptake of DA by a neighboring cell (that

is normally GABAergic in nature) can result in its loss is a testimony to the cytotoxic role of DA.

10.3.3 Is DA Really the Culprit?

In view of the toxic potential of oxidative reactions involving DA, and the experimental evidence supporting the cytotoxic effects of DA oxidation products *in vivo*, it is curious that a considerable regional variability exists in the vulnerability of DA neurons toward degeneration in PD. Notably, the mesencephalon contains two major dopaminergic neuronal populations, namely, the A9 neurons of the SNpc that project to the striatum along the nigrostriatal pathway and the A10 neurons of the ventral tegmental area (VTA) that project to the limbic and cortical areas along the mesolimbic and mesocortical pathways [12]. Despite their anatomical proximity and biochemical and electrophysiological similarities, the A9 neurons are the ones that are selectively lost in PD. Furthermore, this subset of dopaminergic neurons are also lost at a significantly faster rate compared to other neuronal types under conditions of normal aging in the apparent absence of environmental toxin exposure [75]. Although the exact reason for this selectivity remains unclear, Chung and colleagues have recently demonstrated that the expression of antioxidant genes is intrinsically higher in A9 neurons than in A10 neurons [76], suggesting that A9 neurons may be constantly experiencing a sustained heightened level of oxidative stress. Given this, one would assume that the ability of A9 neurons to cope with sudden or chronic increase in ROS production may be more limited than that of their A10 counterparts. Supporting this, treatment of the two neuronal populations with ROS inducers such as MPTP, 6-OHDA, and paraquat (all of which are parkinsonian neurotoxins) results in the death of A9 but not A10 neurons [26, 77, 78]. Conversely, transgenic mice with increased activity of SOD1 or glutathione peroxidase, key ROS scavenging enzymes, are resistant to MPTP-induced dopaminergic neurodegeneration [57, 79].

Why the A9 neurons are in an apparent state of heightened oxidative stress is, however unclear, although a recent study by Mosharov and colleagues demonstrated that cytosolic DA concentrations in these neurons are significantly (2- to 3-fold) higher than in the more resistant VTA neurons [80]. Not surprisingly, cytosolic DA-enriched A9 neurons are found to be more sensitive than A10 neurons to the toxic effects of an acute L-DOPA challenge [80]. The difference in DA levels between these two groups of neurons may be attributed to differences in synthesis (as there is no significant difference with regard to precursor uptake, storage, or degradation of DA), which appears to be

related to the use of distinct calcium (Ca^{2+}) channels by these neurons. Unlike the VTA neurons, A9 dopaminergic neurons use L-type Ca^{2+} channels to help maintain autonomous pacemaking [81]. By virtue of the activity and thereby opening of this type of channel in the absence of synaptic inputs, the A9 neurons experience a significantly larger magnitude and spatial extent of Ca^{2+} influx [82]. Importantly, antagonizing L-type Ca^{2+} channel diminishes the differences in cytosolic DA levels between A9 and A10 neurons and concomitantly reduces the toxic effects of acute L-DOPA treatment in the former group of neurons [80], suggesting a complex interplay between calcium and DA in the degeneration of A9 neurons in PD.

The reliance of SN dopaminergic neurons on voltage-dependent L-type Ca^{2+} channels obviously comes with a price, as intracellular Ca^{2+} concentration is under very tight homeostatic control by the actions of ATP-dependent pumps whose operations are metabolically expensive. A sustained entry of Ca^{2+} in these neurons would presumably work the mitochondria machinery harder and concomitantly raise the level of ROS that would predispose them to oxidative stress-induced degeneration. Supporting this, a recent elegant study conducted by Guzman et al. [83] in transgenic mice showed that the basal oxidation of a mitochondrially localized redox-sensitive form of GFP (mito-roGFP) is indeed significantly higher in SN dopaminergic neurons relative to their VTA counterparts. Furthermore, the level of mito-roGFP oxidation in SN (but not VTA) dopaminergic neurons can simply be lowered by the administration of L-type Ca^{2+} channel antagonists in these transgenic mice. Importantly, the ablation of DJ-1 expression results in the amplification of basal oxidant stress in SN dopaminergic neurons. Collectively, the findings by Guzman et al. emphasize the intimate relationship between L-type Ca^{2+} channels and mitochondrial oxidant stress. This “L-type Ca^{2+} hypothesis” [82] is certainly an attractive proposition, as neurons in the locus ceruleus (LC) that are also lost in the PD brain are similarly autonomous pacemakers dependent on the activity of L-type Ca^{2+} channels [84]. Furthermore, the majority of A10 neurons contain Ca^{2+} -binding proteins such as calbindin D-28K that are capable of buffering intracellular Ca^{2+} to prevent it from rising to damaging levels within the cell [85]. Comparatively, the A9 neurons express calbindin D-28K at significantly lower levels [76]. However, it is noteworthy that PD pathology according to the neuropathological staging proposed by Braak is not confined to the SN or LC but progressively extends from the caudal to the rostral brain regions, involving not just the dopaminergic system but also noradrenergic, cholinergic, and serotonergic systems that may or may not rely on L-type Ca^{2+} channels [86]. Thus, as attractive as the “L-type Ca^{2+} hypothesis” may seem, it does not

adequately explain all the predilection sites in the PD brain, although it remains an extremely attractive factor underlying the vulnerability of SN dopaminergic neurons to degeneration.

10.3.4 Extracellular Oxidative Stress

It is important to recognize that in addition to intrinsic factors, extracellular oxidative stress mediated by activated glial cells may also accelerate the degeneration of SN dopaminergic neurons in disease conditions. In their normal state, glial cells participate in the maintenance of neuronal homeostasis in several ways, including providing trophic support to neurons and helping to clear neurotransmitter released into synapses [87, 88]. Upon their activation during neuroinflammation, reactive astrocytes and microglia release various inflammatory cytokines and complement factors to repair or dispose of damage cells and thereby reestablish the regional microenvironment. Associated with this is the production of ROS and RNS [89]. Although beneficial, excess or chronic neuroinflammation can obviously exert unintended deleterious effects on neighboring dopaminergic neurons and contribute to disease progression [90]. The activation of glial cells is well documented in affected regions of the PD brain, as well as in genetic and toxin-induced models of PD [91]. Glial cell activation is usually accompanied by the upregulation of iNOS, which is either absent or expressed at very low level in the normal brain. Notably, iNOS null mice are less susceptible to MPTP-induced neurotoxicity compared to their wild-type counterparts and, accordingly, display reduced staining for markers of oxidative stress [80]. Similarly, inactivation of the inflammatory enzyme NADPH-oxidase, which catalyzes the production of superoxide from oxygen and NADPH, results in the attenuation of MPTP-induced neurotoxicity in mice [92]. The expression of both iNOS and NADPH-oxidase are elevated in the PD brain [92, 93]. Activated microglia is also known to upregulate the expression of cyclooxygenase-2 (Cox-2) and thereby the synthesis of prostaglandins that could, in turn, kill neurons directly through the activation of caspase-3 or indirectly via the excessive release of glutamate by astrocytes [94]. Interestingly, SN dopaminergic neurons express Cox-2, suggesting that dysregulated Cox-2 levels may lead to cell-autonomous suicide [95]. Inhibition of Cox-2 apparently mitigates the formation of the deleterious DA-quinone and reduced toxicity in a MPTP mouse model [95]. Notwithstanding the obvious damage to neighboring cells that chronic neuroinflammation could bring, the glial response is often taken to be a secondary cause of the neurodegenerative process. Furthermore, neuroinflammation is not limited to PD but a consistent feature

of neurodegenerative diseases in general. Nonetheless, the role of non-cell-autonomous neuronal death is regaining its prominence in the field as more and more researchers are revisiting the cross talk between neurons and glial cells in neurodegeneration.

10.4 THERAPEUTIC IMPLICATIONS

Given the apparently compelling role of oxidative stress in PD pathogenesis, strategies aimed at reducing oxidative stress should in theory mitigate disease progression and provide clinical benefits. Indeed, the success of such strategies will provide the ultimate proof of concept that oxidative stress is a key factor underlying neuronal death in PD. The first controlled clinical trial to evaluate the potential of antioxidants as neuroprotective agents was the Deprenyl and Tocopherol Antioxidative Therapy of Parkinsonism (DATATOP) study [96]. Deprenyl (selegiline) is an irreversible MAO-B inhibitor that is expected to reduce the formation of DOPAC and hydrogen peroxide from oxidative deamination of DA. In so doing, deprenyl would decrease the formation of oxyradicals from hydrogen peroxide generated by DA metabolism. α -tocopherol is vitamin E, an established antioxidant. However, this study together with several other subsequent studies utilizing similar antioxidant strategies failed to produce convincing protection against PD. If oxidative stress is indeed the primary cause of PD pathogenesis, why have these trials apparently failed? There could be several explanations for the generally disappointing outcomes of antioxidant clinical trials in PD, including the study design involved, the availability of administered compounds in the brain, and the heterogeneity of PD patients. Furthermore, as much as we like to believe that oxidative stress is a central player in PD pathogenesis, it is obviously not the only player but one important factor among several others all interwoven in a tapestry of events underlying the degeneration process. To expect a singular therapy to work effectively for a complex disease like PD may just be too tall an order. Perhaps a combination of therapies targeted at major problem centers in the pathogenic cascade may be the way to go, although getting administrative approval for such cocktail drug trials would invariably pose a challenge. A “druggable” target related to the oxidative stress cascade is obviously the L-type Ca^{2+} channel, which can be antagonized by dihydropyridines (DHPs). DHPs have been approved for human use for the treatment of hypertension. Interestingly, a large Danish study conducted recently demonstrated that reducing Ca^{2+} influx in the brain via the administration of DHPs decreases the risk for PD [97]. Given the interplay between DA and calcium in

dopaminergic neurodegeneration, and the availability of FDA-approved Ca^{2+} channel antagonists, it may be worthwhile to explore the clinical benefits of combining antioxidants with Ca^{2+} channel blockers for the PD patient.

10.5 CONCLUSION

It is apparent from the discussion above that oxidative stress has a definite role in PD pathogenesis. Indeed, virtually all the epidemiological PD risk factors identified to date are directly or indirectly linked to oxidative stress. Similarly, the majority of the genetic causative factors of the disease are associated in one way or another with oxidative stress. Even normal aging, recognized as an unequivocal risk factor for PD, is accompanied by increased indices of oxidative stress. Coupling these phenomena to the knowledge that intrinsic pro-oxidant factors like iron, DA, and L-type Ca^{2+} channel all can confer neuronal vulnerability to degeneration, it is hard not to be persuaded by the oxidative stress hypothesis in PD pathogenesis. However, a nagging question that constantly surfaces is whether oxidative stress represents a cause or a consequence of PD. The “chicken and egg” argument here is that oxidative stress can be as much a promoter as it is a result of neuronal death. This is a longstanding debate that is difficult to resolve.

As discussed above, several studies have highlighted an unequal distribution of pro- and antioxidant factors in different regions of the brain [98]. Notably, the basal oxidant level in vulnerable neurons is typically higher than in neighboring neurons that are unaffected, as in the case between A9 and A10 dopaminergic neurons, even in seemingly healthy brain. The most logical assumption to make here is that neurons with high intrinsic oxidant levels are predisposed to degeneration but not necessarily already exhibiting signs of degeneration or undergoing degeneration. The corollary is that oxidative stress is unlikely to be a mere passive consequence of neuronal death, notwithstanding that dying neurons can further elevate pro-oxidative events. A related question that many often ask is whether oxidative stress represents a primary or secondary event in PD. Perhaps a more pertinent question to ask here is whether we should be taking a reductionist approach in pinpointing causative factors of PD. Many would agree that the PD pathogenesis involves an intricate network of interacting pathways rather than a linear sequence of events. For example, oxidative stress can cause mitochondrial dysfunction and vice versa. Likewise, oxidative stress can damage the proteasome, and, conversely, proteasome dysfunction can also lead to oxidative stress [99]. In this circle of events, oxidative

stress can therefore be a primary cause as much as it can represent a secondary cause of the disease. The important thing to recognize is that while oxidative stress is a persuasive key player in PD pathogenesis, it is likely to be one among several other key players.

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OXIDATIVE STRESS IN CARDIOVASCULAR DISEASES

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11.1 INTRODUCTION

Cardiovascular diseases comprise several complex conditions, such as inflammatory, cerebrovascular, ischemic, hypertensive, and rheumatic diseases. Collectively, cardiovascular diseases contribute to 30% of all causes of mortality around the world, whereas ischemic heart diseases alone represent ~13% (Fig. 11.1). Besides the invaluable loss of lives, cardiovascular diseases also contribute to economic burden [1].

As for all complex diseases, there is not a single cause for cardiovascular diseases. However, several factors have been described as risk factors for the development of cardiovascular diseases [2]. Among these are high-fat diet, smoking, alcohol consumption, age, sex, and race. Interestingly, despite the broad range of manifestation, atherosclerotic plaque formation could be considered the common starting point for all cardiovascular diseases. It has a chronic inflammatory component, but its effects may manifest acutely, as stroke or acute myocardial infarction.

Reactive oxygen species (ROS), reactive nitrogen species (RNS), and oxidative stress are the basis of many hypotheses about the development of cardiovascular diseases. Both ROS and RNS have important physiological functions as chemical weapons in immune cells, vascular tone regulators, and signaling molecules [3]. Conversely, ROS and RNS can have roles as bad agents during the onset and progression of cardiovascular diseases. Why does this happen? Mainly because risk factors are supposed to cause imbalance, either favoring ROS/RNS

production or impairing antioxidant mechanisms. Thus it is easy to understand why smoking, which can decrease antioxidant defenses and generate reactive aldehydes, represents a risk factor for cardiovascular diseases [4].

The discovery of the imbalance between ROS/RNS generation and its consumption in cardiovascular diseases led to the false belief that the Holy Grail was found and that antioxidant therapy could be life saving. The truth is that researchers have not found a common ground and the evidence is still controversial, as we shall see further in this chapter.

11.2 FREE RADICALS—ORIGINS AND FATES

Free radicals are molecules or atoms that are able to have a free existence despite having one unpaired electron, which confers a high reactivity. Among the most-studied free radicals are superoxide ($O_2^{\bullet-}$), hydroxyl (HO^{\bullet}), peroxy (RO_2^{\bullet}), and nitric oxide (NO^{\bullet}). Reactive oxygen species (ROS) are a wide class of reactive molecules that includes free radical and other nonradical molecules that are also highly reactive. Also considered ROS are molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), ozone (O_3), and peroxynitrite ($ONOO^-$) [3].

Superoxide is the most abundant free radical in cells. Despite this, it is not highly reactive. Even so, it can act as a signaling molecule or as a substrate for H_2O_2 and peroxynitrite generation. Perhaps the main source of superoxide is the electron transport chain in mitochondria, since it has been estimated that 1–3% of total oxygen is leaked as

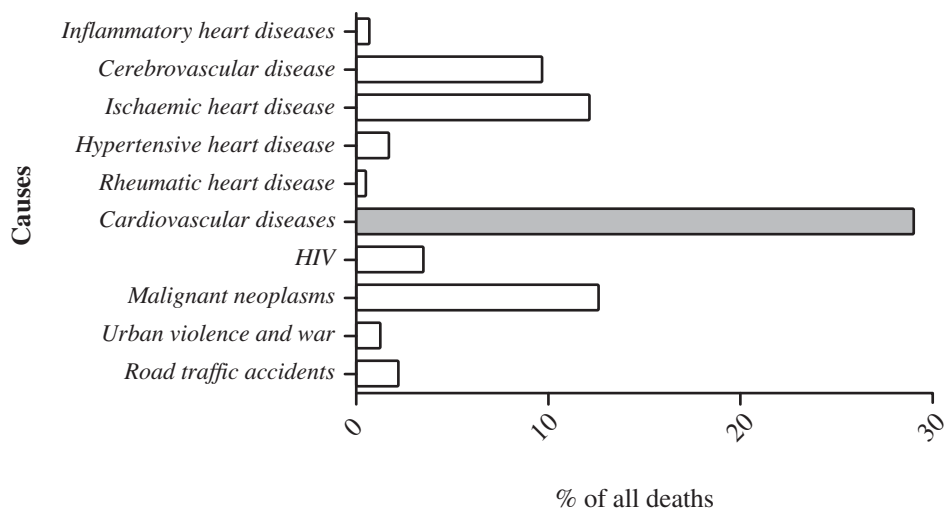


Fig. 11.1 Estimated deaths by cause worldwide. Data from World Health Organization for 2004.

superoxide in physiological processes. NAD(P)H oxidase oxidizes the reducing agent NAD(P)H and transfers the electron to the molecular oxygen, yielding superoxide. It is mainly employed as a chemical weapon by neutrophils and macrophages, but it is highly important in endothelial and myocardial dysfunction [5]. Xanthine oxidoreductase is also an important source of superoxide that oxidizes xanthine or hypoxanthine in uric acid and transfers the electron to NAD^+ rather than to O_2 . In some cases, such as after myocardial infarction, this enzyme suffers spatial modifications that make it an oxidase that transfers the electron to oxygen rather than to NAD^+ (see Fig. 11.2).

Superoxide dismutase is the enzymatic defense against superoxide produced in cells and exists in three isoforms: a manganese-dependent mitochondrial isoform (Mn-SOD), a copper- and zinc-dependent cytosolic isoform (Cu,Zn-SOD), and an extracellular tetrameric

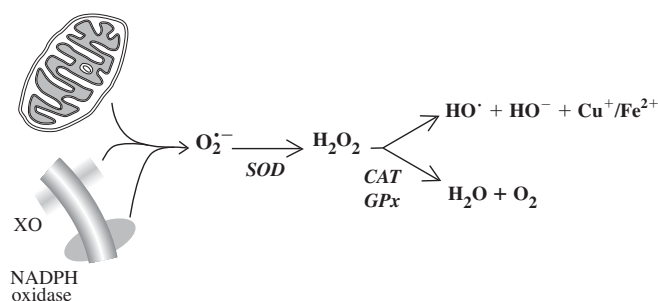


Fig. 11.2 The three main sources of superoxide radical in cells are mitochondria, xanthine oxidase (XO), and NAD(P)H oxidase. After superoxide production, a chain of coupled enzymatic reactions leads to the consumption of reactive oxygen species to avoid oxidative stress. In the case of hydrogen peroxide accumulation, the reaction with transitional metals generates the most harmful radical, hydroxyl.

Cu,Zn-dependent isoform (EC-SOD). These enzymes act on superoxide, and the product is hydrogen peroxide, according to reaction (1) in Figure 11.3.

Although hydrogen peroxide is not a free radical, it is important as a signaling molecule, since it is highly diffusible. Moreover, the reaction of hydrogen peroxide with transitional metals (e.g. Cu^+ , Fe^{2+}) yields the most reactive and harmful radical in biological systems, hydroxyl. To avoid hydroxyl generation, the antioxidant enzymes such as catalase and glutathione peroxidase (GPx) are able to use hydrogen peroxide to generate water and molecular oxygen, according to reactions (2) and (3) in Figure 11.3, respectively. This chain of events is depicted in Figure 11.2.

NO is a diffusible gas that has important physiological roles. The source of NO can be any of the three isoforms of nitric oxide synthase NOS): neuronal (nNOS), endothelial (eNOS), or inducible (iNOS). NOS converts the amino acid L-arginine into NO and another amino acid, L-citrulline. Certain cofactors are needed, such as FAD, FMN, and tetrahydrobiopterin (BH_4). NOS can also produce superoxide in the presence

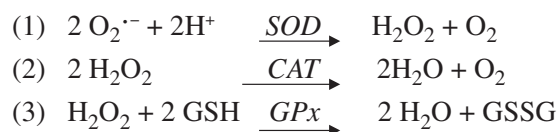


Fig. 11.3 The main enzymatic antioxidant system is shown. Equation (1) represents the action of superoxide dismutase (SOD) on superoxide radical. Equation (2) represents the action of enzyme catalase (CAT) on SOD product hydrogen peroxide. Equation (3) represents the action of glutathione peroxidase (GPx) on the same hydrogen peroxide. The difference is the necessity of reduced glutathione (GSH) as cofactor.

of low levels of BH_4 , which may contribute to atherosclerosis generation, as we shall see. NO exerts its signaling effects binding to iron-heme groups in guanylate cyclase. The signaling effects are best comprehended as antihypertensive, since NO can stimulate cGMP synthesis in smooth muscle of blood vessels and thus induce relaxation and lower blood pressure. Besides its physiological roles, NO can react with superoxide to give peroxynitrite. This nonradical ROS is able to oxidize reduced cysteine, lipids, and DNA. The most-studied damage in proteins is the modification of tyrosine to 3-nitrotyrosine.

To summarize, in case of any disturbance between free radical production and its consumption by the antioxidant system, harmful species can arise. These species are prone to react with any molecule, such as DNA, protein, and lipids, which may lead to errors in transcriptional activity, signaling, and membrane permeability. When this damage overcomes the capability of the organism for repairing it, oxidative stress takes place.

11.3 CARDIOVASCULAR DISEASES

Although cardiovascular diseases might vary in their symptoms, endothelial dysfunction, hypertension, and atherosclerosis appear to be at the core of all cardiovascular complications. The formation of atheromatous plaques can increase blood pressure and vice versa. Narrowing of blood vessels may lead to ischemic events that increase free radical production that damages cells, contributing to endothelial and cardiac dysfunction. From development to progression, redox imbalance and subsequent oxidative stress play a crucial role in all these diseases.

11.3.1 Atherosclerosis

Atherosclerosis is characterized by thickening of the arterial wall due to macrophage infiltration, smooth muscle migration, and cholesterol deposition. Symptoms can occur late in the course of atherosclerotic plaque formation and are mainly due to the narrowing of vascular lumen or to the rupture of plaque, which leads to the obstruction of a small capillary. Regardless of the type of manifestation, the final event is the decrease of blood supply to the organ. Initiation and progression of atherosclerosis involve complex mechanisms, and many aspects are still not understood.

Again, the participation of free radical in atherosclerotic plaque formation seems to be a unifying theory, since all known risk factors contribute to ROS/RNS generation. For example, F_2 -isoprostanes, a well-accepted marker of

lipid oxidation, nitrated proteins, and lipid hydroperoxides, are all associated with increased risk for developing atherothrombotic diseases. Moreover, several stimuli that in some way participate in atherosclerotic plaque formation also trigger NAD(P)H oxidase-mediated superoxide production [6]. For instance, fluid shear stress, the frictional force generated by blood flow over the vascular endothelium, is a major factor in atherogenesis. Branched and curved arteries are considered prone to lesion, whereas straight arteries are less prone. Interestingly, cultured endothelial cells subjected to shear stress *in vitro* produce twice as much superoxide and monocyte binding than cells subjected to laminar stress. Also, this effect does not occur in cells deficient in p47 protein, a component of NAD(P)H oxidase complex [7].

NO can be synthesized constitutively by endothelial isoform of NOS (eNOS) or, after an induction, by the inducible isoform (iNOS). The former participates in vascular tonus regulation, antagonism of platelet aggregation, and leukocyte binding, whereas the latter is involved in the proinflammatory process [8]. The inducible isoform is detected in human atheroma as soon as monocyte infiltration and foam cell differentiation starts [9]. Also, it was recently shown that iNOS colocalizes with other proatherosclerotic factors (ACE and AT_1) whereas eNOS vanishes in endothelial cells from autopsy [10].

Despite being considered essential as antiatherosclerotic, eNOS deletion in mice is not enough to induce plaque formation because nNOS compensatory activity can keep NO production in a normal range [11]. It is noteworthy that eNOS can undergo a process called uncoupling in which the reduction of molecular oxygen is no longer coupled with L-arginine oxidation. Thus eNOS generates superoxide rather than NO [12]. Among several factors that could lead to eNOS uncoupling, BH_4 deficiency and presence of asymmetrical dimethyl arginine (ADMA) appear to be the main factors. BH_4 acts by improving L-arginine binding and donating the second electron to oxygen reduction. Thus, in the absence of BH_4 , eNOS still partially reduces oxygen without oxidizing L-arginine, generating superoxide [12]. ADMA may act as an eNOS inhibitor by competitive inhibition of L-arginine oxidation or may uncouple eNOS activity. In fact, it has been found that coronary artery disease patients carrying the low-activity form of the rate-limiting enzyme in BH_4 synthesis (GTP cyclohydrolase I) produce more superoxide and oxidized low-density lipoprotein (oxLDL) and demonstrate diminished response to acetylcholine induced vasorelaxation [13]. Plasma levels of ADMA were inversely related to acetylcholine-induced vasorelaxation in saphenous veins collected from patients undergoing coronary bypass surgery, whereas it was directly related to superoxide production by both saphenous vein and internal mammary artery [14].

Oxidative stress can have two main consequences: First, after oxidizing catalytically active or structural biomolecules, it can lead to a loss or gain of function and thus precipitate some dysfunction. Second, oxidatively modified molecules become a new class of molecules, and some of them can bind to membrane receptors and trigger signals. A good example is the increased level of oxidation of fibrinogen seen in patients with coronary artery disease. Formation of NO-derived species can lead to 3-nitrotyrosine formation in fibrinogen. The consequence is an accelerated and aberrant fibrin polymerization that presents resistance to lysis [15–17].

In addition to oxidative modification, a process called glycation also occurs in diabetes and is believed to contribute to the generation of atherosclerotic plaque. Briefly, glycation consists in a series of processes that involve a nonenzymatic reaction between a reducing sugar (e.g., glucose) and amino groups of proteins to form a reversible Schiff base. This reaction is prone to some arrangements, and free radicals are part of this complex process. After that, a new and heterogeneous class of molecules called collectively advanced glycation end-products (AGEs) arises [18]. Among the epitopes generated are *N*^ε-(carboxymethyl)lysine (CML), pentosidine, and GA-pyridine. CML has been extensively studied and has been associated with a variety of inflammatory processes. Immunohistochemical studies have already found CML and GA-pyridine in human atherosclerotic lesions both intracellularly in foam cells and in the extracellular environment [19, 20].

Whether AGE formation is a consequence of an inflammatory process or it has any active role in atherosclerotic plaque generation seems not to be under debate nowadays. AGEs constitute a class of signaling molecules that exert their effects mainly through receptor for AGE (RAGE). RAGE is a transmembrane receptor belonging to the immunoglobulin superfamily that acts as a multiligand receptor [21]. Among the RAGE ligands are AGEs, S100/calgranulin proteins, high mobility box group 1 protein (HMGB1), and oxLDL [22]. One of the best characterized consequences of RAGE activation is the stimulation of free radical-generating pathways, which in turn will lead to the generation of additional AGEs and oxLDL. Moreover, RAGE stimulation triggers intracellular signaling that culminates with NF- κ B translocation to the nucleus, which is largely described as a transcriptional factor associated with proinflammatory response [23]. In line with this, diabetic mice lacking RAGE showed less incidence of atherosclerotic plaque formation in aorta than mice expressing RAGE [24]. The central role of RAGE in atherosclerosis development suggests that this receptor should be seen as a therapeutic target in that condition. In fact, this suggestion has already been tested in animal models with good results

[25], but unfortunately there are no current clinical trials testing this approach.

Environmental factors are well-established risk factors for atherosclerosis development, and many of them are linked to oxidative stress generation inside the body or are themselves reactive species. Cigarette smoking is one of the most important and modifiable risk factors associated with the generation and progression of cardiovascular diseases [26]. Cigarette smoke contains high concentrations of free radicals, such as superoxide, NO, carbon-centered radicals, and other toxic compounds, such as acrolein and formaldehyde. Even passive smokers have increased risks for cardiovascular diseases that are mediated by increased oxidative stress [27]. Guinea pigs subjected to cigarette smoking for 28 days develop atherosclerosis, which could be avoided with dietary vitamin C supplementation [28]. This rationale indicates that free radicals are important in the onset and progression of atherosclerotic plaque.

However, antioxidant supplementation in humans (smokers and nonsmokers) has yielded contradictory results. Vitamin E supplementation (800 IU) seems to be protective in a cohort of atherosclerotic patients, decreasing the risk of nonfatal myocardial infarction by half [29]. Despite this, a study with a large cohort followed up for 6 years demonstrated that vitamin E alone has no effect on atherosclerosis progression [30]. Even so, when associated with vitamin C, the antioxidant therapy was able to diminish the intima-media thickening in the common carotid artery of smoker subjects [30].

Diet is another source of toxic compounds that could contribute to atherosclerosis development. AGEs can be generated during the cooking process, and after ingestion they can reach the circulatory system and participate in some mechanisms. Diabetic patients with a poor AGE diet presented decreased levels of circulatory AGE, VCAM, and C-reactive protein compared with a regular diet [31]. The relation between dietary AGEs and atherosclerosis is better understood when looking at preclinical models. For instance, mice subjected to femoral artery injury and fed with low-AGE diet presented less neointima area and larger luminal area than the high-AGE diet group. These data reinforce the role of AGEs in neointima proliferation after vascular lesion [32].

11.3.2 Heart Infarction and Heart Failure

The heart is a highly oxidative organ, and fatty acid oxidation is responsible for 60–80% of its energy requirement [33]. Because of this oxidative metabolism, 25–35% of total cardiomyocyte content is occupied by mitochondria [34]. Even under physiological states (normoxia), 1–3% of oxygen is leaked as free radicals by

mitochondria, and this value can be increased several-fold during or after infarction.

Acute myocardial infarction is defined as an interruption in the blood flow in myocardium. The most common cause is the occlusion of coronary artery following the rupture of an atherosclerotic plaque, and the obvious consequence is the death of starved cells. During the infarction, oxidative stress is caused both by inflammatory cells that infiltrate and by cardiomyocytes after the onset of necrosis. There is no doubt that the ischemic process must be stopped and that the reestablishment of blood flow is necessary to avoid acute heart failure. This can be done through pharmacological thrombolytic therapy or surgically, and these interventions help to decrease the mortality rates after acute myocardial infarction [35]. It turns out that this procedure is considered a “double-edged sword” since it is as harmful as it is necessary and results in “ischemia-reperfusion injury” (IRI). The clinical manifestation of IRI is seen as myocardial stunning, arrhythmia, myocyte death, and endothelial/microvascular dysfunction [36]. Several intricate steps occur after IRI, and free radicals act centrally in this process. Chronic treatment with β -blockers, aspirin, angiotensin-converting enzyme (ACE) inhibitors, and statins have improved the survival and the quality of life in infarcted patients. However, myocardial infarction remains a prevalent event with devastating consequences since it causes loss of cardiomyocytes, which are terminally differentiated cells. With few myocytes remaining it is hard to sustain adequate contractile function and heart failure develops.

11.3.3 The Ischemic Phase

During the ischemic process low levels of oxygen and nutrients arrive in the heart. The only way to keep the levels of ATP for contractile function is by increasing the glycolysis rate, which will lead to an overload of lactate and acidosis. As the glycogen vanishes, there will be a fall in ATP levels and an increase in its degradation products (hypoxanthine). These changes in energetic balance are the main cause of mitochondrial permeability transition (MPT) pore opening [37]. The MPT pore is a voltage- and Ca^{2+} -dependent high-conductance channel, whose opening leads to the increased permeability of inner mitochondrial membrane with consequent dissipation of the electrochemical proton gradient that drives mitochondrial functions. Also, MPT pore opening promotes the release of free radicals and cytochrome *c*, a proapoptotic factor that, once in cytosol, triggers caspase-3 and the apoptotic process [38]. The increase in permeability caused by MPT pore opening causes an influx of water into the mitochondrion, which makes it swell. The final step is the rupture of this organelle.

When the insult is severe, there will be neither time nor ATP for the cell to enter the apoptotic process, and necrosis will occur. In fact, it was recently confirmed by atomic force microscopy that mitochondrial swelling happens in infarcted rat hearts [37].

11.3.4 The Reperfusion Phase

Prolonged periods of ischemia are related to necrosis, whereas reperfusion is related to the start of apoptosis. The reestablishment of glucose and oxygen restores the ATP levels necessary for apoptosis but also restores the levels of molecular oxygen needed to generate free radicals. Then, as soon as the reestablishment of blood flow takes place, the IRI starts. The ischemic process that precedes reperfusion represents a priming phase in oxidative stress. The ischemic process itself is not free radical generating, since it causes the lack of the main substrate, oxygen. However, as a priming phase, it causes changes in xanthine oxidase (as discussed above), in antioxidant enzymes and in several mitochondrial proteins, which predisposes the cell to an oxidative burst as soon as oxygen enters the cell [39, 40].

All of the mechanisms involved in IRI are not well known, but the overproduction of ROS, mainly by mitochondria and xanthine oxidase, has been related to IRI. During the ischemic process ATP levels fall and the ADP formed is also further oxidized to AMP, adenosine, hypoxanthine, and xanthine. The enzyme responsible for the last two steps is xanthine oxidoreductase that is present in the organism as the reductase isoform. Xanthine reductase oxidizes xanthine and transfers the electrons to NAD^+ instead of to O_2 . During an insult process, such as heart infarction, the ion gradient disruption leads to Ca^{2+} accumulation and activation of Ca^{2+} -stimulated proteinases. In the reoxygenation process, oxidation of $-\text{SH}$ groups or limited proteolysis of xanthine reductase converts it to an oxidizing isoform, and now the oxidation of xanthine also leads to the monovalent reduction of molecular oxygen, generating superoxide anion [3].

In fact, since 2003 a clear relationship has been recognized between high serum uric acid and mortality in patients with chronic heart failure [41]. Probably uric acid in serum represents the increased xanthine oxidase activity and, eventually, increased oxidative stress in those patients. In light of these data, some studies have attempted to link the use of allopurinol with decreased mortality in heart failure patients. For instance, allopurinol administration in patients enrolled for revascularization caused less cardiac damage, measured by creatine phosphokinase and troponin I release. Moreover, patients receiving allopurinol showed 13% less incidence of death, infarction, or tachycardia in a 30-day follow-up [42].

Perhaps a major role in IRI is developed by mitochondria. Besides being a source of free radicals in the reperfusion phase, it is also a depository of proapoptotic molecules. Thus any dysfunction would lead to oxidative damage and cell death. Isolated hearts subjected to IRI have provided information about free radical generation in a more confident way, since data from isolated mitochondria may present some bias regarding the isolation process and in vivo data are hard to obtain. In this way, it was evidenced by electron paramagnetic resonance (EPR) that ubisemiquinone, alkyl-peroxy radical, and nitrogen-centered radical were generated after the reperfusion [43]. The mitochondrial source of ubisemiquinone and alkyl-peroxy radical could be confirmed in hearts perfused with sodium amobarbital, an inhibitor of complex I of the mitochondrial electron transport chain. The major consequence was a decreased membrane lipid oxidation and high pressure developed by hearts at the end of reperfusion [43].

An early target of mitochondrial free radicals is cardiolipin, a phospholipid located inside the inner mitochondrial membrane that is responsible for attaching cytochrome *c*. The oxidation of this phospholipid is related to a decreased electron transport activity, increased free radical generation, and cytochrome *c* release with eventual caspase-9 activation [44]. Genetic approaches and pharmacological studies confirm that cardiomyocyte apoptosis plays a crucial role in the pathogenesis of many cardiac syndromes and pathologies. For instance, the inhibition of cardiac myocyte apoptosis reduces infarct size up to 50–70% and decreases cardiac dysfunction after IRI [45, 46].

The impairment of mitochondrial complex I, III, and IV activity due to ROS-induced cardiolipin damage may increase the electron leak from the electron transport chain, generating more superoxide anion radical and perpetuating a cycle of oxygen radical-induced damage, which ultimately leads to a decrease in oxidative phosphorylation and to heart failure on reperfusion. Perhaps a good strategy should be to reperfuse with low pO_2 in order to try to attenuate free radical generation, as demonstrated in isolated rat hearts. This strategy improved mitochondrial electron transport, decreased H_2O_2 production, and improved cardiolipin oxidation compared with normoxic reperfused hearts [47].

Antioxidant enzyme modulation (e.g., SOD, catalase, GPx) seems not to contribute largely to the oxidative damage in IRI. Some studies showed that Mn-SOD decreases after IRI [48], whereas other studies showed no modulation in Mn-SOD, EC-SOD, and Cu,Zn-SOD content [49, 50]. Moreover, IRI also promoted a decrease in catalase and GPx in an in vivo model of transient ischemia [51]. Taken together, these data suggest that the increase in free radical production without a consistent compensation

of the antioxidant machinery contributes to oxidative damage in IRI. Artificially increasing antioxidant enzymes by transgenic overexpression or supplementing with exogenous enzymes/vitamins/antioxidants may contribute to limit the damaged area. These data suggest that antioxidant supplementation during reperfusion could avoid IRI and render better results over the long term.

11.3.5 Cardiac Remodeling

Cardiac remodeling occurs in response to a variety of stimuli, such as hypertension or myocardial infarction. It represents functional and structural changes, and crucial aspects are involved in remodeling: ventricular hypertrophy, development of interstitial fibrosis, loss of contractility, and chamber dilation. As the signs worsen, heart failure takes place. These changes are associated with an extensive inflammatory process and are, to some extent, necessary to replace the damaged myocardium. As remodeling progresses, it becomes harmful by compromising normal cardiac function [52].

11.3.6 Hypertrophy

ROS production is present in a variety of animal models of cardiac hypertrophy (response to α -adrenergic agonists, angiotensin II stimulation, and cyclic stretch) [53]. Free radicals have an important part in coordinating the cardiac remodeling, and NAD(P)H oxidase seems to be an important source. A study with mice lacking subunit gp91^{phox} (Nox2) of NAD(P)H oxidase showed that there was no hypertrophic response to angiotensin II in those mice [5]. In contrast, mice lacking p91^{phox} were not protected against hypertrophy by aortic banding (a model of response to pressure overload), which seemed to be compensated by an increase in expression of another related subunit of NAD(P)H oxidase, Nox4. The role of free radicals in hypertrophy was further confirmed by treating these mice with the antioxidant *N*-acetylcysteine, which could prevent the hypertrophic response [54]. Superoxide anion appears to be central in the free radical-guided hypertrophy, since NAD(P)H oxidase modulation can avoid hypertrophic response. The participation of other ROS in cardiac hypertrophy can not be neglected, since *N*-acetylcysteine is a nonspecific antioxidant.

11.3.7 Interstitial Fibrosis

The deposition of an excess of extracellular matrix components between cardiomyocytes contributes to decreased cardiac elasticity and electrical conductance (see Section 11.3.10 Arrhythmia). The proliferation of myofibroblasts is another key component of interstitial fibrosis.

NAD(P)H oxidase also participates in collagen I and III deposition in both perivascular space and left

ventricular (LV) tissue. Rats rendered hypertensive by aldosterone treatment showed lower levels of collagen I and III when treated with apocynin, an NAD(P)H oxidase inhibitor [55]. Superoxide from other sources, such as xanthine oxidase, could also participate in this process. Infarcted mice develop extensive cardiac remodeling, but allopurinol, a xanthine oxidase inhibitor, could reverse collagen deposition in myocardium and preserve echocardiographic parameters, such as ejection fraction and chamber diameter [56].

11.3.8 Contractility

Multiple mechanisms contribute to contractile dysfunction, including abnormalities of cardiomyocyte excitation-contraction, mitochondrial dysfunction and energetic deficit, loss of myocytes from the ventricular walls, and alterations in the extracellular matrix. Interestingly, NAD(P)H oxidase has a role in contractility of cardiomyocytes independent of other effects that also interfere in contractility (chamber dilation and fibrosis). Cardiomyocytes isolated from mice lacking gp91^{phox} and wild-type mice subjected to aortic banding clearly demonstrated that NAD(P)H oxidase absence improved contractility independent of other effects on the tissue [57]. Contractility has been shown to be preserved when another source of superoxide is blocked. Allopurinol, an inhibitor of xanthine oxidase, was able to preserve cardiac contractility in hearts from dogs with dilated cardiomyopathy [58]. The downstream effects of superoxide may involve the generation of other ROS and regulation of sarcolemmal ion channels, sarcoplasmic reticulum calcium release channel (the ryanodine receptor), sarcoplasmic reticulum calcium pump (SERCA 2a), and the contractile proteins themselves [53, 59].

11.3.9 Chamber Dilation

Matrix metalloproteinases (MMPs), a family of enzymes that catabolize collagen, elastin, and gelatin, and their tissue inhibitors (TIMPs) are central during cardiac remodeling. Several MMPs become activated in heart failure [60]. In animal models, it has been demonstrated that deletion of MMPs is accompanied by reduced LV [61] dilation and inflammation after myocardial infarction. Conversely, deletion of TIMPs is accompanied by increased LV dilation after myocardial infarction (MI) [62]. Free radical participates in activation of MMPs. Mice subjected to a model of pressure overload by aortic constriction present increased levels of superoxide and MMP-2 activation. Mice lacking iNOS did not present MMP-2 activation or suffer all these adverse remodeling effects on the heart. Such effects could also be avoided by the selective iNOS inhibitor administered

in wild-type mice subjected to aortic constriction [63]. In the same line, infarcted hearts from mice that were treated with allopurinol presented attenuated chamber dilation compared with those who were treated with saline [56]. Moreover, other studies have demonstrated that heart-specific deletion of Mn-SOD promoted a progressive dilated cardiomyopathy [64]. Collectively, these data suggest the importance of free radicals in the progression of chamber dilation.

11.3.10 Arrhythmia

Arrhythmia is defined as any change in the electrical activity in the heart. It is classified according to the alteration produced. Tachycardia consists of accelerated heartbeat, while bradycardia is characterized by a slower pump rate. Although events of arrhythmia can result in cardiac arrest and sudden death, some arrhythmias occur as sparse events and are not considered as threats. The most important causes of arrhythmia are coronary artery disease and atrial fibrillation (AF), the latter being closely associated with postsurgery inflammation.

11.3.11 Atrial Fibrillation

AF is characterized by rapid electrical activity of the atria that leads to abnormal ventricular activity [65]. One general cause of AF is heart failure (HF), although intrinsic mechanisms vary and are not well understood. Besides HF, diabetes mellitus and cardiac surgery increase the risk of AF [66].

Animal models have shown that increased atrial pacing is associated with increased superoxide production. In addition, most of this radical is produced by the enzyme NAD(P)H oxidase [67]. In humans, NAD(P)H oxidase activity is a strong predictor of postoperative AF [68]. The process of atrial fibrosis itself is closely related to the activation of the AT₁ receptor by angiotensin II and the subsequent increase in NAD(P)H oxidase activity. Inhibition of AT₁ is known to prevent superoxide production [69].

Along with free radical production, inflammation is considered as a risk factor and also a component of AF. Postoperative high levels of interleukin 6 (IL-6) are predictors of AF onset [70]. Moreover, serum levels of hydroperoxides are associated with atrial function and predict AF in patients after radiofrequency catheter ablation [71].

Antioxidants have been proven as efficient in treatment and prevention of AF and arrhythmias, although their role is no more than that of coadjutants. The pool of glutathione (GSH) is a major physiological defense against oxidative stress in the myocardium. Indeed, its precursor *N*-acetylcysteine was shown to decrease the

incidence of postischemia arrhythmias [72, 73]. The use of drugs such as thiazolidinediones might act by reducing the activity of xanthine oxidase or by increasing NO production. This NO is capable of reacting with the overproduced superoxide radical, due to the increased activity of NAD(P)H oxidase. Ascorbic acid also seems to be an important antioxidant, as its levels were found decreased in a dog model of atrial tachypacing [74], and its administration to patients with persistent AF reduced the recurrence of the condition after cardioversion [75]. NO precursors such as L-arginine and sodium nitropruside have strong potential as therapeutics, since NO acts as an antioxidant and anti-inflammatory [76, 77].

Although their origins might rely on other cardiovascular complications such as heart failure and atherosclerosis, AF and arrhythmias are strong life-threatening conditions with specific mechanisms of development that require specific interventions.

11.4 EXERCISE AND PROTECTION AGAINST FREE RADICAL-MEDIATED CARDIOVASCULAR DISEASES

As stated above, environmental factors contribute greatly to increased risk of cardiovascular diseases. Among these are smoking and dietary habits (see above). Another important habit that contributes to cardiovascular diseases is lack of physical activity. Physical activity can either confer resistance to cardiovascular events, such as myocardial infarction, or help during the recovery process.

11.4.1 Exercise as a Protector

There is a large amount of epidemiological evidence showing that physical activity habit has a protective effect for several cardiovascular complications, such as atherosclerosis development and hypertension [78]. But does exercise also work for improving life after the myocardial infarction episode? The answer seems to be positive, since men who had physical activity after an episode of myocardial infarction were more prone to survive [79, 80]. Unfortunately, direct evidence of protective effect of physical activity is hard to obtain, thus we can rely on animal models to understand the mechanism involved. From these models we can learn that exercise preconditioning exerts its effects by decreasing the size of infarct [81], a parameter that is closely linked with the mortality rate in humans [82]. But how does exercise decrease mortality of cardiomyocytes after an infarction? The following effects are involved: first, morphological changes concerning myocyte hypertrophy and increased coronary vascularization; second, changes in metabolic efficiency; and third, increase in

the myocardium antioxidant potential after a transient release of free radicals.

ROS such as hydrogen peroxide and superoxide were already described as important signaling molecules in a variety of processes, including myocyte hypertrophy and death. Isolated cardiac myocytes subjected to mechanical stretch present increased levels of superoxide and develop hypertrophy [83]. These events are mediated by mitogen kinases, such as ERK1/2, which is implicated in cell growth and can be stimulated by free radicals in cardiac myocytes. When antioxidants are given to these cells, not only superoxide is abolished, but also ERK1/2 activation and cell growth [83]. The participation of ROS in myocyte hypertrophy becomes clearer with the demonstration that hydrogen peroxide delivery to cultured adult cardiomyocytes has a biphasic response, being proliferative in low doses and apoptotic/necrotic at higher doses [84]. Lower doses of H₂O₂ (10–30 μ M) induce activation of ERK1/2 and cell growth, whereas higher doses (>100 μ M) induce activation of ERK1/2, JNK, and p38 MAPK, and JNK activation is related to the deleterious effects of H₂O₂ treatment [84].

From studies with animals we can learn that high-intensity exercises can increase MnSOD activity in both young and old rat hearts [85, 86], indicating that the heart retains the ability to induce antioxidant defense despite age. Interestingly, the cytoplasmic isoform of superoxide dismutase (Cu,ZnSOD) is, in general, not induced by physical exercise [87]. The chemical product of SOD action is hydrogen peroxide, and it serves as substrate for catalase and glutathione peroxidase [3]. Thus these hydrogen peroxide-consuming enzymes should be kept in balance with the SOD increment to avoid an excess of peroxide and a possible start of oxidative stress. However, it seems that the exercised heart proceeds otherwise, since there is no compensatory increase in catalase or glutathione peroxidase [87]. Thus, taken collectively, these data indicate that the increase in H₂O₂ in cardiac myocytes during the exercise can lead to myocyte growth and thus to cardioprotection against future events.

Besides this, free radicals are centrally involved in angiogenesis. Vascular endothelial growth factor (VEGF) is a family of key mediators of angiogenesis after wound or exercise that exert their effects through VEGF receptors (VEGFR1, VEGFR2, and VEGFR3) [88]. A well-conducted study in animals showed that the delivery of low doses of H₂O₂ to the wound can increase VEGF mRNA and CD31⁺ cells and accelerate wound healing [89]. Moreover, either NAD(P)H oxidase deficiency or overexpression of catalase abolished VEGF synthesis and impaired wound healing [89]. These data are consistent with the impaired wound healing seen in chronic granulomatous disease patients, who also present dysfunctional NAD(P)H oxidase [90]. In the same way,

antioxidant therapy is considered antiangiogenic, and some studies have employed it as adjuvant therapy to cancer treatment. Several compounds, such as glutathione, selenium, and resveratrol have been shown to impair angiogenesis [91–93].

But how does hydrogen peroxide induce VEGF synthesis? The pathway is not completely elucidated yet, but some studies employing different cell types have shown that the MAPK pathway, mainly involving MEK, ERK1/2, and p44/42 MAPK, seems to be important [88]. Moreover, VEGF signaling is further stimulated because hydrogen peroxide also triggers NF- κ B translocation to the nucleus and stimulates the transcription of VEGF receptors [88].

Finally, physical activity increases the antioxidant potential in the heart in order to counterbalance the permanent increase in free radical generated as a consequence of heart work. Thus, in the case of a myocardial infarction, the heart is more prone to buffer the oxidative burst during the reperfusion process. However, this view is not fully elucidated, as we shall see below. First, there are some studies with chronically exercised animals that do not show any improvement in the heart antioxidant profile [94]. SOD, for instance, appears to be upregulated only after high-intensity exercise [87]. Also, exercise induced MnSOD activity in both young and old rats [86]. Moreover, the systems responsible for consuming hydrogen peroxide do not respond after exercise in most studies [87]. Glutathione increase after exercise is also under debate, and its role in heart adaption is not fully elucidated. In fact, because hydrogen peroxide acts as signaling molecule in heart adaption, we should not to expect an increase in the systems responsible for consuming it.

11.4.2 Exercise as a Therapy

After the onset of myocardial infarction a lot of attention is devoted to decreasing the remodeling process that takes place and leads to heart failure. The remodeling process involves cardiomyocyte death, fibroblast proliferation, and extracellular matrix deposition [52], all processes coordinated in some way by free radicals. Besides the classical therapies, exercise training may attenuate cardiac remodeling and may even reverse this process [95]. Unfortunately, few studies have focused on the effect of exercise on free radical after myocardial infarction. In one study, 17 patients were enrolled in a training program for 3 weeks. At the end, the patients presented better hemodynamic performance and lesser hydrogen peroxide production after the physical test [96]. Similar results were found in another study that evaluated antioxidant enzymes in skeletal muscle biopsies from chronic heart failure patients who entered into a physical activity

program [97]. Despite these studies, which clearly showed that physical activity performed after a myocardial insult is efficient in restoring free radical/antioxidant balance, there has been no evaluation of the effect of exercise on survival rate in a long-term study.

11.5 ANTIOXIDANT THERAPIES FOR CARDIOVASCULAR DISEASES

Atherosclerosis is one of the leading causes of morbidity and mortality in Western countries. Its occurrence is in great part explained by the way of life in a technologically advanced world. Lack of exercise activities and high-fat diet are major risk factors for atherosclerosis. Indeed, evidence suggests that diets low in saturated fat and rich in vegetables contribute against the development and progression of cardiovascular diseases [26]. It is clear that antioxidants present in the diet play an important role by acting against the intrinsic mechanisms of atherosclerosis and other cardiovascular diseases, but clinical trials and follow-up studies are controversial [98].

Studies with animals often support antioxidant therapies to prevent or attenuate atherosclerosis. A low-vitamin C and E diet accelerated atherosclerosis in ApoE-deficient mice [99]. In the same model of atherosclerosis, *N*-acetylcysteine, a precursor of glutathione, reduced plaque formation and superoxide production [100]. Although it may seem fairly obvious that antioxidants can prevent cardiovascular diseases, tests in humans raise many questions about the specificity of each antioxidant and of each disease. Furthermore, some clinical studies with vitamins A and E report an increase in mortality and incidence of cardiovascular disease [101–103].

On the other hand, some antioxidants have been demonstrated to be effective. After infarction, administration of L-arginine has been proved to ameliorate the response to the ischemic event by increasing the levels of NO, which promotes vasodilation and can also neutralize reactive species. Besides NO production, L-arginine also increases the activity of SOD and the levels of thiols, and both are believed to act synergistically with the vasodilation [104]. Potent free radical scavengers like edaravone have also been tested in humans and proven to improve LV ejection fraction and reduce rehospitalization after infarction [105]. However, it is clear that no antioxidant will be found to prevent all cardiovascular diseases or restore cardiovascular function after any deleterious event.

Some success has been achieved using xanthine oxidase inhibitors, such as allopurinol and oxypurinol. Patients with ST segment elevation myocardial infarction who underwent reperfusion showed better recovery markers when treated with allopurinol than those treated with placebo. Allopurinol was able to completely recover

ST elevation in 76% of the patients, against only 58% in placebo-treated subjects. Moreover, markers of myocyte damage, such as cardiac troponin I and CK-MB, were also lower in patients treated with allopurinol [42]. Also, congestive heart failure patients with ejection fraction <40% receiving oxypurinol over a month demonstrated 7% of improvement in ejection fraction compared with the placebo group [106]. Thus xanthine oxidase indeed seems to be important in the course of heart failure, and superoxide may be a therapeutic target in the future.

In this sense, antioxidants are important agents in prophylaxis and treatment, regulating the intra- and extracellular redox status. Fine-tuning of the antioxidant enzymes and production of glutathione is associated with lower incidence of cardiovascular diseases and increase of life span. However, while the specific mechanisms lying behind cardiovascular diseases are still to be elucidated, the use of antioxidants in medicine might remain underrated.

11.6 CONCLUSION

Oxidation of biomolecules accelerates cell senescence and is closely associated with neurodegenerative and cardiovascular diseases. As the organism gets older, antioxidant defenses and repair mechanisms become weaker and cannot counteract the production of reactive species and accumulation of oxidative damage. Although this scenario seems cruel, fine regulation of the oxidative status of cells and tissues is a key point in the maintenance of homeostasis. Regarding the cardiovascular system, oxidative stress shows up as a major culprit, participating in endothelial dysfunction, development and progression of atherosclerosis, and cardiac dysfunction. In any case, reducing the production of reactive species or increasing the levels of antioxidants has proven to be important for prevention and treatment. Nevertheless, clinical trials are inconclusive and sometimes controversial. From this perspective, better knowledge of the mechanisms regulating oxidative stress generation, as well as new therapeutic interventions, capable of acting more specifically in each disease, can be foreseen.

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OXIDATIVE STRESS AND AGING: A COMPARISON BETWEEN VERTEBRATES AND INVERTEBRATES

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12.1 INTRODUCTION

Aging and death, the two ubiquitous phenomena, have always been an enigma for mankind. Except for certain primitive organisms such as bacteria that propagate through simple division, all organisms including humans must age and subsequently face death, yet their life span can vary. Among major model organisms, the worm *Caenorhabditis elegans* lives for 2 weeks, the fly *Drosophila melanogaster* lives for 2 months, the mouse *Mus musculus* lives for 2 years, and humans live for approximately 80 years [1].

Old age in most species is associated with impaired adaptive and homeostatic mechanisms leading to susceptibility to environmental/internal stresses with increasing rates of disease and death [2]. Since ancient times humans have pondered over the mysterious question: How do we age? How do different organism/species show different life spans, and what are the factors that determine longevity? Hippocrates (460–377 B.C.) defined aging as an irreversible and actual event dictated by the gradual loss of heat. Darwin established the scientific background to understand the process of aging; he explained aging as the loss of excitability over time.

The term “aging” refers to the biological process of growing older in a deleterious sense, which some authors call “senescence” [3, 4]. It is a unique feature of the life cycle of all organisms. In scientific terms, aging may be defined as an inevitable process of accumulation of molecular, cellular, and organ damage, leading to loss

of function and increased vulnerability to disease and death [5].

12.2 THEORIES OF AGING

Aging is a multifactorial process caused by damage by a variety of cellular components, followed by their accumulation. It is characterized by progressive deterioration of physiological functions and metabolic processes [6, 7]. Within an organism, manifestation of aging occurs at genetic, molecular, cellular, organ, and system levels [8]. Long-term studies on numerous animal models (in vivo and in vitro) have generated a number of theories that attempt to elucidate the cause/mechanism(s) of aging, since it is doubtful that a single theory can explain all the mechanism of aging. Presently more than 300 theories of aging are in existence [9].

The evolutionary theory of aging states that aging occurs because the force of natural selection declines with age in populations, making it possible for hazardous late-acting genes to exist [10, 11]. The life history principle describes aging as an emergent phenomenon that takes place primarily in the protected environment and that allows survival beyond the natural life span that would occur in the wild. The natural or essential life span (ELS) of a species is the time required to fulfill the Darwinian purpose of life, that is, successful reproduction. The period of extended survival beyond the ELS is defined as the period of aging [12]. The mutation accumulation theory argues that detrimental, late-acting mutations

may accumulate in the population and ultimately lead to pathology and aging [13]. According to the energy consumption hypothesis, animals are born with a limited amount of potential energy or physiological capacity, the faster they use it, the faster they will die [14]. Later this hypothesis became the rate of living theory: The faster the metabolic rate, the faster the biochemical activity, the faster an organism will age. The protein error theory also tried to explain the mechanism of aging. According to this theory an induction and increase in protein errors can accelerate aging in human cells and bacteria [15–17]. Similarly, the accuracy of protein synthesis can slow aging and increase the life span in fungi [18, 19].

The cellular senescence/telomere theory explains that cells have a limited proliferative potential. After a finite number of divisions, cells enter into a state of senescence, and this process of cell senescence limits the number of cell divisions [20]. The number of divisions a cell can undergo in culture is known as the Hayflick limit and has been postulated to determine the maximum life span of an organism [21, 22]. Furthermore, it was elaborated that this specific type of cellular senescence results from the loss of a small amount of DNA at the end of the chromosome, resulting in ever shorter telomeres. This limit in the replicative capacity results in terminally arrested cells with altered cellular physiology that might contribute to aging and cancer through secondary effects on neighboring cells in tissue [23, 24].

The role of genes in regulation of longevity has also been put forward; this theory postulates that aging results from changes in gene expression [25–27]. The free radical theory of aging, developed by Denham Harman in 1956, is one of the most influential theories in describing the aging mechanism. According to this theory free radicals, specifically hydroxyl and hydroperoxyl, are formed endogenously from normal oxygen-utilizing metabolic processes as by-products that play a essential role in aging and age-related processes. Mitochondria play a central role in generation of free radicals through impaired function of the electron transport chain, and these free radicals elicit damaging properties. Aging results in accumulation of free radical damages as a function of time [28–31]. Support for the free radical theory of aging has increased progressively over the years, and growing numbers of studies implicate free radical reactions in aging and the pathogenesis of specific diseases such as diabetes, cancer, and heart diseases [32–34].

12.3 FREE RADICAL/OXIDATIVE STRESS THEORY OF AGING

Among several theories that attempt to explain the aging mechanism, the free radical/oxidative stress theory of

aging offers the best mechanistic elucidation of the aging process and other age-related events [28, 35].

Interest in the free radical theory was at first very limited because of persistent doubt about the existence of oxygen free radicals in biological systems despite the reports by Gerschman et al. [36] and the detection of radicals by Commoner and co-workers [37]. The discovery of superoxide dismutase (SOD) by McCord and Fridovich [38] and the demonstration of the existence of H_2O_2 in vivo by Chance [39] gave credibility to and raised the profile of the hypothesis. In 1972, Harman modified his free radical theory, ascribing a central role to mitochondria [29] because mitochondria generate a disproportionately large amount of free radicals/reactive oxygen species (ROS) in cells [39]. Later on, correlative evidence supporting the free radical theory of aging was published. Later on, several reports substantiated Harman's hypothesis that free radical oxidative damage increases during aging [40–42].

In the last few decades, Harman's hypothesis has been refined; it is now accepted that not only free radicals but also other forms of activated oxygen such as peroxides and aldehydes (which are not technically free radicals) play a role in oxidative damage in cells. A collective term, "reactive oxygen species (ROS)," has been introduced to define these oxidants including free radicals. This realization led to a modification of the free radical theory, that is, the oxidative stress theory of aging [43].

Apart from respiratory chain in mitochondria, there are other endogenous sources of ROS including immune reactions, enzymes such as xanthine oxidase and nitric oxide synthase, and transition metal-mediated oxidation [44]. A diverse range of exogenous sources of ROS are reported, which incorporate ionizing and nonionizing radiations, pollutants, natural toxic gases such as ozone, drugs, and toxins including oxidizing disinfectants [34, 45]. ROS production and accumulation is a common denominator in many diseases and environmental insults and can lead to severe cellular damage resulting physiological dysfunction and cell death in virtually all aerobes. When oxidative stress occurs, cells function to counteract the oxidant effects and to restore redox balance by resetting critical homeostatic parameters. Such cellular activity leads to activation or silencing of genes encoding defensive enzymes, transcription factors, and structural proteins [34, 46]. According to the free radical theory of aging, oxidative stress increases with increasing age; this condition leads to accumulation of oxidation products of lipids, nucleic acids, proteins, sugars, and sterols ultimately causing cellular dysfunction and making the body prone to external deleterious agents (Fig. 12.1). In agreement with the free radical/oxidative stress theory of aging, it was investigated that mtDNA deletions are induced by oxidative stress and dramatically accumulate with age in organisms ranging from worms to humans [40, 47].

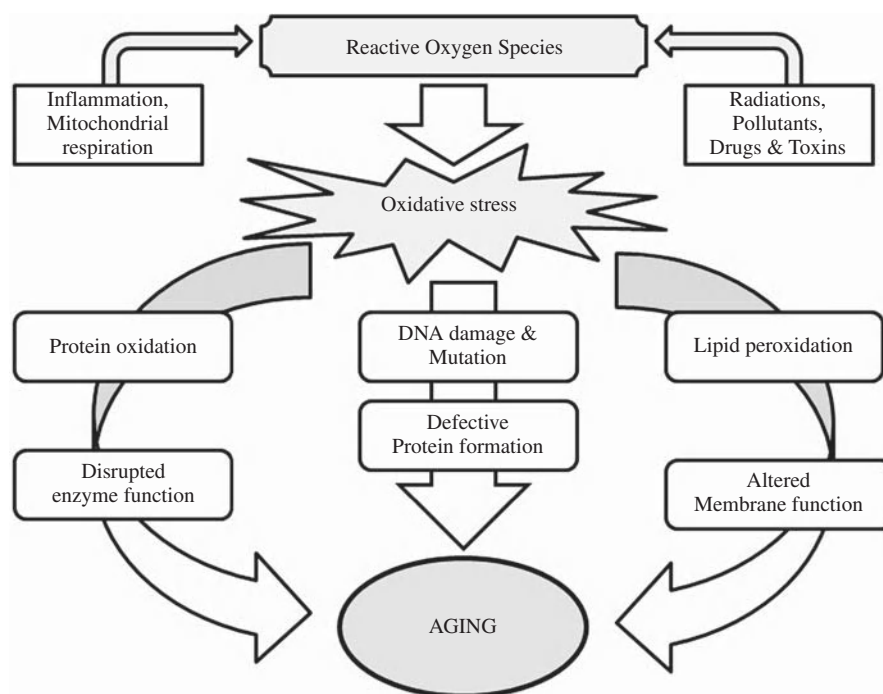


Fig. 12.1 Reactive oxygen species generated by endogenous as well as exogenous sources cause oxidative damage and accumulation of proteins, lipids and DNAs, when defensive mechanisms of body become weak. These disturbances cause organelle damage, changes in gene expression followed by altered cellular responses which ultimately results into aging.

12.4 AGING IN INVERTEBRATES: ROLE OF OXIDATIVE STRESS

The role of oxidative stress in aging is clearly seen in *Podospora anserina*, a fungus that belongs to the ascomycete family of fungi. The growth of hyphae in *P. anserina* is not indeterminate; however, its growth arrests on reaching a certain length and hyphae eventually wither and die. The period to onset of the condition of senescence is referred to as the life span of *P. anserina*. Tudzynski and Esser in 1979 stated the central involvement of mitochondria in determining the timing of senescence [48]. Recently, Dufour et al. [49] proved the involvement of ROS in life span limitation of *P. anserina* and demonstrated that elimination of the mitochondrial electron transport chain extended the hyphal life span of *Podospora* by at least threefold. It has been proposed that oxidative damage to mitochondrial DNA by the mitochondrial ROS triggers senescence [40, 50, 51].

Saccharomyces cerevisiae, the common yeast, is the other invertebrate whose life span is directly determined by oxidative stress/ROS. *S. cerevisiae* also belongs to the ascomycete family. This single-cell budding yeast is a good organism for studying the aging process. For multiplication *S. cerevisiae* uses asymmetric cell division, which results in a small daughter cell; the process is known as budding. Interestingly, a yeast cell (mother

cell) can only bud a finite number of times, after which it loses its capacity to multiply and becomes sterile. This budding period of the yeast cell is called the clonal life span [40].

Studies have revealed oxidative damage accumulation during clonal life span. Accumulation of oxidatively damaged proteins has been reported in yeast mother cells during this period [52]. The attack by ROS against proteins modifies amino acid residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as a measure of protein damage [53]. In yeast cells, protein carbonyls have been shown to accumulate during chronological aging, in a manner dependent on the rate of mitochondrial ROS production [54]. Besides overexpression of the methionine oxidation repair enzyme, MsrB and MnSOD have been unambiguously shown to increase chronological life span of yeast cells [40, 55]. Collectively, these data suggest that ROS limit life span during aging in *S. cerevisiae*; however, the role of oxidative stress in determining the clonal and chronological life span in yeast cells is still not fully understood.

The nematode *Caenorhabditis elegans* is one of the most frequently used invertebrate models for studying the aging process because of its many interesting characteristic features ideally required to study mechanism(s) of aging. Large numbers of offspring, short generation

time, and the ability to be stored frozen have attracted the attention of biogerontologists. Unlike the unicellular yeast, it allows studies of different cell types and organs, such as the nervous or digestive systems; it is also more closely related to mammals [5]. During the past few years, a large number of long-lived mutants of *C. elegans* have been identified that are now frequently used as models in aging research. The commonly used mutants of *C. elegans* are *daf-2* and *age-1* [56, 57]. The *daf-2* gene encodes a protein with structural similarity to the mammalian insulin and the insulin-like growth factor receptors, while the *age-1* gene encodes a catalytic subunit of phosphatidylinositol-2-OH kinase, which is involved in a conserved signal transduction pathway downstream of the insulin-like receptor [56]. Studies on *daf-2* reveal that these mutants show resistance to oxidative stress, elevated antioxidative enzymes, and expression of some antioxidant genes; all these strongly support the oxidative stress hypothesis of aging [58, 59]. Several recent studies report that there is indeed slower age-related accumulation of protein carbonyl groups in long-lived *C. elegans* strains compared to wild type [60, 61] and faster accumulation in a short-lived strain [61].

In support of the free radical theory in the aging process, Lithgow and co-workers showed that catalytic antioxidants like catalase (CAT) and SOD markedly extend the life span of *C. elegans* [62]. Although some reports do contradict the oxidative stress theory in *C. elegans* aging, there is enough evidence for oxidative stress as a life span determinant in *C. elegans* [40].

The free radical theory of aging has also been extensively studied in the fruit fly (*Drosophila melanogaster*). The fly is important for establishing evolutionary conservation of mechanisms and for studying events in different tissues that are more numerous and differentiated than in *C. elegans*. Early experiments have indicated that there is a quasi-linear, inverse relationship between life span and oxygen tension in *Drosophila*, and it was seen that increase in oxygen tension above 21% shortened the life span [63, 64]. In 2004, Landis et al. [65] in their microarray study compared gene expression patterns in old and young flies by treating young flies with 100% oxygen. The results were interesting: Young flies treated with 100% oxygen exhibited many gene expression changes resembling those in old flies. This indicated that oxidative injury plays a prominent role in normal fly aging and suggests that the life span shortening under mild hyperoxia (40% O₂) may be true accelerated aging [40].

Genetic manipulation of the SOD activity in *Drosophila* emphasizes the direct association between oxidative stress and life span. Increasing the activity of different forms of SOD by two- to fourfold suggests that elevated antioxidant defense is necessary for

extended longevity in *Drosophila* [66]. In 1995, Sohal et al. reported that concomitant overexpression of SOD and CAT increased both average and maximum life span of *D. melanogaster* [67]. Parkes et al. also targeted the overexpression of CuZnSOD in *Drosophila* and reported that overexpression of CuZnSOD in motor neurons resulted in an increase in life span as well as an increase in resistance to paraquat and γ -irradiation [68]. Phillips and co-workers in 2000 reported that MnSOD overexpression increased life span in *Drosophila* [69].

Some recent transgenic studies provide more evidence for the oxidative stress theory of aging in *Drosophila sp.* Overexpression of the protein oxidative damage repair enzyme peptide-S-methionine sulfoxide reductase (MsrA) was found to increase average life span in several independent insertion lines [70]. Many contradictory reports have been published that argue the role of oxidative stress in the aging process of *Drosophila sp.* Nevertheless, there is a fair amount of evidence to show the involvement of ROS as a limiting factor in determining the life span of *D. melanogaster* [40].

In addition to the above-discussed invertebrate models there are many other invertebrate species such as helminths in which the direct influence of oxygen radicals in longevity determination has been reported [40].

12.5 AGING IN VERTEBRATES: ROLE OF OXIDATIVE STRESS

Vertebrates are an evolved group of animals that include mammals and humans. Invertebrate model organisms have been used for the discovery of genes and mechanisms involved in extension of life span, but the mouse is the most practical mammal for establishing whether homologous genes and processes can extend healthy life span [5]. In vertebrate models caloric restriction has been shown to increase life span. Caloric restriction also protects against age-related decline in function and disease in rodents and monkeys, and in humans it reduces risk factors for diabetes, cardiovascular disease, and cancer [5]. The most accepted explanation behind caloric restriction life extension is based on the free radical theory of aging: The rate of mitochondrial free radical production is directly proportional to the oxidative damage. A decreased rate of operation of mitochondrial electron transport chain leads to a lower oxidative burden and a higher life span [71].

Studies on transgenic mice have been carried out to understand the relation between free radical generation, oxidative stress, and longevity. Takagi and co-workers in 1999 reported an increase in life span of mice after approximately threefold overexpression of human thioredoxin 1 (*hTrx1*) in different tissues [72]. An increased

resistance to cerebral ischemia and to UV-induced oxidative stress was also demonstrated in *hTrx1* mice. Mitsui et al. in 2002 documented that *hTrx1* mice lived about 35% longer than littermate control mice. In addition, a 22% increase in maximal life span was also observed [73]. In continuation, transgenic mice overexpressing metallothionein have been shown to have an increased mean life span of about 15% relative to control mice. Metallothionein is a low-molecular-weight protein rich in cysteine residues; it exhibits heavy metal detoxification and free radical scavenging abilities [74].

Transgenic mice that overexpress human catalase in peroxisomes, nucleus, or mitochondria have also been tested [76]. The mitochondrial overexpression of catalase (MCAT) in mice caused significant delay in the development of age-related cataracts. This was an interesting finding in support of the free radical theory of aging since the development of age-related cataracts has been shown to be inversely related to life span in humans, and its incidence is reduced in mice that exhibit extended longevity [40, 75]. It was seen that Ames dwarf mice have an increased life span compared to their wild-type littermates [76]. Further investigations revealed that the dwarf mice have increased levels of CAT and SOD [77, 78]. The Ames dwarf mice also showed reduced levels of DNA and protein oxidation in liver [78]. Embryonic fibroblasts from these mice are resistant to UV radiation, heat stress, paraquat, hydrogen peroxide, and cadmium [56, 79].

In addition, interspecies comparisons of the life span in vertebrates show a very clear role of oxidative stress in determining longevity. In 1999, Kapahi et al. performed comparative experiments to measure the resistance of fibroblasts and lymphocytes from hamster, rat, marmoset, rabbit, sheep, pig, cow, and human to various oxidative stresses such as paraquat, sodium arsenite, hydrogen peroxide, and *tert*-butyl hydro-peroxide (tBHP). Interestingly, they observed that cells from species with a longer life span had increased resistance to oxidative stress and proposed that cellular defenses that help cells to survive acute stresses are associated with longevity [80]. These observations are consistent with the free radical/oxidative stress theory of aging.

In humans there is an array of experimental evidence in agreement with the free radical/oxidative stress theory of aging [16, 34]. Over the past 10 years, major developments have been made in the assay of oxidative damage to lipids, DNA, and protein to measure specific types of oxidative damage in specific types of cells/tissues during aging. Several reports document the age-dependent increase in lipid peroxidation, protein oxidation, and nucleic acid damage in humans, and there exists a significant negative correlation with antioxidant capacity of plasma [34, 81–83]. Overexpression of

antioxidative enzymes/defense mechanisms during aging in humans has also been reported in recent studies that support the oxidative stress theory of aging [83–85].

12.6 CONCLUSION

The phenotype and the rate of progression of aging are highly variable in different species and in organisms within a species. Although there is no doubt that aging is a multifactorial process that is governed by more than one factor, it is becoming increasingly clear that oxidative stress and the resultant damage to biomolecules play a vital role in several age-related pathologies. Despite some contradictory reports, there is sufficient experimental evidence to conclude that oxidative stress, if not alone, is one of the main limiting factors for aging and life span in invertebrates and vertebrates under normal atmospheric conditions.

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OXIDATIVE STRESS-MEDIATED SIGNALING PATHWAYS BY ENVIRONMENTAL STRESSORS

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13.1 INTRODUCTION

Oxidative stress in the form of excess reactive oxygen species (ROS) or reactive nitrogen species (RNS) can affect cells deleteriously or beneficially. Such stress might be generated by intracellular or extracellular sources. Furthermore, oxidative stress can cause various biological effects. Environmental stress is a key contributor to human disease. A number of substances such as metals, particulate materials, smoke, pesticides, and physical agents are environmental stressors [1] that contribute to many diseases. Concerns related to environmental stressor-related diseases such as cancer, chronic lung disease, diabetes mellitus, neurodegenerative diseases, and reproductive disorders have been raised recently. Research efforts elucidating the modes by which environmental stressors influence the development and progression of diseases or exploring preventive approaches are expected to engender further improvements in our knowledge. Understanding environmental stressor-induced influences at the molecular level will also provide a wealth of information related to the exploration of biomarkers for environmental stressor-related diseases [2–4].

The mechanisms of redox adaptation in living bodies and cells might involve multiple influences on an active redox-sensitive signaling pathway, such as ROS metabolism and antioxidant defenses, p53 pathway signaling, nitric oxide (NO) signaling pathway, hypoxia signaling, transforming growth factor (TGF)- β -bone morphogenetic protein (BMP) signaling, tumor necrosis factor (TNF)

ligand-receptor signaling, and mitochondrial function (Table 13.1). For example, transcription factors such as nuclear factor- κ B (NF- κ B), nuclear factor erythroid 2-related factor 2 (Nrf2), c-Jun, and hypoxia-inducible factor-1 (HIF-1) engender increased expression of antioxidant molecules such as superoxide dismutase (SOD), catalase, thioredoxin, and the GSH antioxidant system. Metal ions such as arsenic (III/V) or copper (II) directly influence expression levels of those transcription factors and induce various oxidative stress events including thiol molecule perturbation, generation of oxidative DNA adducts, and induction of oxidative molecular biomarkers [5–8]. Nonmetal chemicals such as retinoic acids and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are also known to influence the expression of oxidative stress-related genes and proteins during carcinogenesis and during embryonic development [9–12]. In relation to cancer, a growing tumor might also produce intracellular and extracellular oxidative stress, which can modify its malignant features. Endogenous sources of tumor ROS or RNS include impaired intracellular genomes or proteomes, metabolism pathways, and xenobiotic metabolism. Consequently, the study of transcriptional regulation of gene expression in the research field of oxidative stress has been useful for identifying new transregulatory factors or new biomarkers induced by exposure to environmental stressors.

Microarray technology has been used in environmental toxicology and biology studies and has led to the establishment of gene expression signatures profiling

TABLE 13.1 Summary of oxidative stress-mediated signaling pathways

Categorical pathways	Canonical Pathway (orthology)
Reactive Oxygen Species (ROS) Metabolism and Antioxidant Defenses	Glutathione Peroxidases (GPx) Peroxiredoxins (TPx) Superoxide Dismutases (SOD) Genes Involved in Superoxide Metabolism Genes Involved in ROS Metabolism Other Peroxidases and Antioxidant-Related Genes
p53 Signaling (including DNA damage)	Apoptosis-Related Genes Cell Cycle Arrest and Checkpoint Regulation of the Cell Cycle Regulation of Cell Proliferation, Cell Growth, and Differentiation Damaged DNA Binding Mismatch, Base-Excision and Double-Strand Break Repair
Nitric Oxide (NO) Signaling Pathway	Genes with NO Synthase and Regulators of NO Biosynthesis Genes regulated by NO and NO Signaling Pathway Genes Involved in Superoxide Release Antiapoptosis Genes Genes with Antioxidant and Superoxide Dismutase Activity Genes with Glutathione Peroxidase, Oxidoreductase, or Peroxidase Activity Transcription Regulators
Hypoxia Signaling	Response to Hypoxia and Signal Transduction, Oxidative Stress Genes Related to Stress and Immune Response Hemoglobin Complex-Associated Genes Peroxidase, Oxidoreductase-Related Genes Transcription Factors and Regulators and Protein Binding Antiapoptosis Induction of Apoptosis and Caspase Activity Protein Biosynthesis, Phosphorylation, and Metabolism Cytoskeleton and Other Extracellular Molecules Cell Cycle, Cell Proliferation, and Growth Factors Carbohydrate, Lipid, One-Carbon Compound Metabolism RNA Metabolism Cardiac Excitation-Contraction (E-C) Coupling
TGF- β -BMP Signaling	TGF- β superfamily, bmp (bone morphogenetic protein) family members, gdf (growth differentiation factor), activin, and activin receptors Smad family members, TGF- β /activin-responsive genes, bmp-responsive genes, molecules regulating signaling of the TGF- β superfamily, adhesion molecules, extracellular matrix structural constituents, other extracellular molecules, transcription factors and regulators
Tumor Necrosis Factor (TNF) Ligand-Receptor Signaling	Caspase activation, caspase inhibition, anti-apoptosis genes, induction of apoptosis, other apoptosis-related genes, jnk signaling pathway, nfkb signaling pathway, tnfr1 and tnfr2 signaling pathway, inflammatory response, transcription regulators
Mitochondria	Mitochondrial processing, mitochondrial transportation Fatty acid biosynthesis

the toxicity of environmental stressors [13, 14]. Statistical methods used for DNA microarray studies are mostly multivariate approaches. Although basic methods treat genes as traits, which is consistent with the rules of experimental design, several approaches have been developed using expression ratio data sets. Such approaches regard the genes as cases and the array plates as variables. Most well-known methods based on singular value decomposition have used principal component analysis [15, 16]. In alternative approaches, our previous reports have described that a Bayesian

network technique, which is a probabilistic graphical model that represents a set of variable identities, is applicable to investigation of the gene expression interaction networks and the detection of differences arising in them from exposure to different doses of chemicals [17, 18]. Bayesian network techniques can provide predictive information related to the relations between agents and gene expression signatures in the life science fields [19–21].

This chapter addresses various environmental stressor-induced toxicities in experimental animals such as

rats and humans to elucidate the molecular mechanisms underlying toxicity-induced oxidative stress.

13.2 OXIDATIVE STRESS-MEDIATED SIGNALING PATHWAYS

Cells respond and adapt to environmental signals such as stressors [22–24] through multiple mechanisms that involve communication pathways and signal transduction processes. The impact of oxidative stress on various diseases and aging has been reviewed comprehensively. In particular, free radical-induced oxidative stress plays an important role in cancer development, metabolism-related diseases like diabetes and hypertension, and neurodegenerative disorders [4, 25–36]. Our survey of microarray databases and many other published references has revealed the categorical pathways induced by oxidative stress presented in Table 13.2.

ROS metabolism and antioxidant defenses center upon ROS, which are necessary for biological functions and which regulate many signal transduction pathways by directly reacting with and modifying the structure of proteins, transcription factors, and genes to modulate their functions. Actually, ROS induce expression levels of genes associated with signaling cell growth and differentiation, regulating the activity of enzymes (such as ribonucleotide reductase and peroxidase). Control of ROS levels is achieved by balancing ROS generation with their elimination through ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2, and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, and thioredoxin catalase. The ROS can modulate the activities and expression of many transcription factors and signaling proteins that are involved in stress response and cell survival through multiple mechanisms. Therefore, this category includes glutathione peroxidases (GPx), peroxiredoxins (TPx), superoxide dismutases (SOD), genes involved in superoxide metabolism such as arachidonate 12-lipoxygenase (ALOX12), and copper chaperone for superoxide dismutase (CCS). In fact, p53 signaling plays a central role in coordinating the cellular responses to a broad range of cellular stress factors; p53 functions as a node for organizing whether the cell responds to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy. Moreover, p53 controls transactivation of target genes, which is an essential feature of stress response pathways [37–39]. In other words, p53 activation leads to a complicated network of responses to the various stress signals encountered by cells [40–44]. The mitochondrial respiratory chain produces NO, which can generate other RNS when cells are under hypoxic conditions. Although excess ROS and

RNS can engender oxidative and nitrosative stress, moderate to low levels of both function in cellular signaling pathways. Especially important are the roles of these mitochondrion-generated free radicals in hypoxic signaling pathways, which have important implications for cancer, inflammation, and various other diseases [25, 45]. Hypoxic signaling events include vasodilation, modulation of mitochondrial respiration, and cytoprotection following ischemic insult. These phenomena are attributed to the reduction of nitrite anions to NO if local oxygen levels in tissues decrease [46], which activates the expression of genes through oxygen-sensitive transcription factors including HIF and NF- κ B. Hypoxia-dependent gene expression can have important physiological or pathophysiological consequences for an organism, depending upon the cause of the hypoxic insult [47]. These NO signaling and hypoxia signaling pathways are linked to the p53 pathway [48], because recent studies have shown that HIF2 α inhibition promotes p53-mediated responses by disrupting cellular redox homeostasis, thereby permitting ROS accumulation and DNA damage [49]. Reportedly, hypoxia activates the tumor suppressor protein p53 by upregulating Sema3E expression [50].

TGF- β -BMP signaling is involved in developmental morphogenesis and cancer morphogenesis. Morphogens such as those of the TGF- β family inhibit and stimulate basic cell proliferation, respectively, at high and low concentrations. A signaling gradient of declining TGF- β concentration regulates the inhibition and stimulation of cell proliferation [51]. ROS can activate TGF- β either directly or indirectly via the activation of proteases. In addition, TGF- β itself induces ROS production as part of its signal-transduction pathway. Pulmonary tissues are vulnerable to the toxic effects of inhaled air. The oxidant pathways are especially relevant in the lung, where TGF- β is known to have a role in tissue repair and connective tissue turnover. In pulmonary fibrosis and renal endothelial cells, TGF- β activation is considered a hallmark of disease progression [52, 53]. In ovarian cancer, overexpression of FOXG1 contributes to TGF- β resistance through inhibition of p21WAF1/CIP1 expression, which is repressed by p53 [54]. Recent studies have revealed some additional novel functions of the p53 pathway. These include the downregulation of two central cell-growth pathways, the IGF/AKT-1 and mTOR pathways, and the upregulation of the activities of the endosomal compartment [55–57]. The mTOR pathway including the IGF-1/AKT pathway plays critical roles in regulation of cell proliferation, survival, and energy metabolism to shut down cell growth and division to avoid the introduction of infidelity into the process of cell growth and division [58, 59]. In response to stress, IGF-BP3, PTEN, TSC2, AMPK beta1, and Sestrin1/2 are transcribed by p53, play a critical

TABLE 13.2 Oxidative stress-mediated signaling pathways and their related genes

Categorical Pathways	Gene Name (Symbol)
Canonical Pathway (ontology)	
Reactive Oxygen Species (ROS) Metabolism and Antioxidant Defenses	
Glutathione Peroxidases (GPx)	GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, GPX7, GSTZ1
Peroxiredoxins (TPx)	PRDX1, PRDX2, PRDX3, PRDX4, PRDX5, PRDX6
Other Peroxidases	CAT, CSDE1, CYGB, DUOX1, DUOX2, EPX, GPR156, LPO, MGS2, MPO, PIP3-E, PTGS1, PTGS2, PXDN, PXDNL, TPO, TTN
Other Antioxidants	ALB, APOE, GSR, MT3, SELS, SRXN1, TXNDC2, TXNRD1, TXNRD2
Superoxide Dismutases (SOD)	SOD1, SOD2, SOD3
Other Genes Involved in Superoxide Metabolism	ALOX12, CCS, CYBA, DUOX1, DUOX2, GTF2L, MT3, NCF1, NCF2, NOS2A, NOX5, PREX1, PRG3
Genes Involved in ROS Metabolism	AOX1, BNIP3, EPHX2, MPV17, SFTPD
Oxidative Stress-Responsive Genes	ANGPTL7, ATOX1, CAT, CCL5, CSDE1, DGKK, DHCR24, DUSP1, EPX, FOXM1, GLRX2, GPR156, GSS, KRT1, LPO, MBL2, MPO, MSRA, MTL5, NME5, NUDT1, OXR1, OXSR1, PDLIM1, PIP3-E, PNKP, PRDX2, PRDX5, PRDX6, PRNP, RNF7, SCARA3, SELS, SEPP1, SGK2, SIRT2, SRXN1, STK25, TPO, TTN
p53 Signaling Pathway	
Induction of Apoptosis	BAX, BID, CDKN1A, CRADD, EI24, FADD, FASLG (TNFSF6), FOXO3, PCBP4, PRKCA, TNFRSF10B, TP53, TP73, TP73L
Antiapoptosis	BCL2, BCL2A1, BIRC5, CASP2, HDAC1, IGF1R, MCL1, NFKB1, RELA, TNF, TNFRSF10
Other Apoptosis Genes	APAF1, BRCA1, CASP9, E2F1, GADD45A, GML, LRDD, P53AIP1, SIAH1, SIRT1, TP53BP2, TRAF2
Cell Cycle Arrest	CDKN1A, CDKN2A, CHEK1, CHEK2, GADD45A, GML, MYC, PCAF, PCBP4, RPRM, SESN1, SESN2
Cell Cycle Checkpoint	ATR, BRCA1, CCNE2, CCNG2, CDKN2A, RB1, TP53
Negative Regulation of the Cell Cycle	BAX, BRCA1, CDKN2A, MSH2, NF1, PTEN, RBI, TP53, TP73, TP73L, TSC1, WT1
Regulation of the Cell Cycle	BRCA2, CDC2, CDC25A, CDK4, E2F1, E2F3, HK2, IGF1R, KRAS, PPMID, PRKCA, STAT1, TADA3L, TP53BP2
Other Cell Cycle Genes	BIRC5, CCNH, CCNB2, ESR1, MLH1, PCNA, PRC1
Negative Regulation of Cell Proliferation	BAI1, BCL2, BTG2, CDKN1A, CDKN2A, CHEK1, GML, IFNB1, IL6, MDM2, MDM4, NF1, PCAF, PPMID, SESN1
Positive Regulation of Cell Proliferation	IGF1R, IL6
Cell Proliferation	BRCA1, CDC25A, CDC25C, CDK4, E2F1, MYC, PCNA, PRKCA
Cell Growth and Differentiation	ESR1, MCL1, MYOD1
Other Genes Related to Cell Growth, Proliferation, and Differentiation	EGR1, FOXO3A, JUN, KRAS, PTTG1
DNA Repair Genes	ATM, ATR, BRCA1, BTG2, CCNH, DNMT1, GADD45A, MSH2, PCNA, PTTG1, TP53, XRCC5
Human Nitric Oxide Signaling Pathway	
Genes with Nitric Oxide Synthase or Oxidoreductase activity	NOS1, NOS2A, NOS3, NQO1
Positive Regulators of Nitric Oxide Biosynthesis	HSP90AB1 (HSPCB), INS
Negative Regulators of Nitric Oxide Biosynthesis	DNCL1, GLA, IL10

Other Genes Involved in NO Biosynthesis	AKT1, ARG2, DDAH2, DNCL1, EGFR, GCH1, GCHFR
Genes Induced by NO	CDKN1A, IL8, JUN, VEGFA
Genes Suppressed by NO	CCNA1, MYB, TROAP
Genes Involved in NO Signaling Pathway	CAMK1, DLG4, GRIN2D, NOS1, PPP3CA, PRKAR1B, PRKCA
Genes Involved in Superoxide Release	ALOX12, DUOX1, DUOX2, NOX5, PRG3
Genes with Oxidoreductase Activity	ALOX12, CYBA, DUOX1, DUOX2, NOS2A, NOX5, SOD1, SOD2, SOD3
Genes with Peroxidase Activity	DUOX1, DUOX2
Genes with Superoxide Dismutase Activity	SOD2
Other Genes Involved in Superoxide Metabolism	CCS, NCF1, NCF2, PREX1
Antiapoptosis Genes	MPO, MTL5, NME5, PRDX2, RNF7
Genes with Antioxidant Activity	APOE, MT3, SELS, SOD1, SOD3, SRXN1 (C20orf139)
Genes with Glutathione Peroxidase Activity	GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, LOC493869
Genes with Oxidoreductase Activity	CAT, EPX, GPX1, GPX2, GPX3, GPX4, GPX5, GPX6, LPO, MPO, MSRA, PRDX2, PRDX6, SOD1, SOD2, SRXN1(C20orf139), TPO, TXNRD2
Genes with Peroxidase Activity	CYGB, EPX, GPR156, LPO, MPO, PRDX2, PRDX5, PRDX6, TPO, TTN, UNR
Transcription Regulators	FOXO1, GLRX2, SIRT2, SIRT2, SOD2, UNR
Other Genes Involved in Oxidative Stress	ATOX1, DUSP1, GSS, KRT1, MBL2, NUDT1, OXR1, PNKP, PRNP, SCARA3, SEPP1, SGK2
DNA Damage Signaling	
Apoptosis	ABL1, BRCA1, CIDEA, GADD45A, GADD45G, GML, IHPK3, PCBP4, AIFM1 (PDCD8), PPP1R15A, RAD21, TP53, TP73
Cell Cycle Arrest	CHEK1, CHEK2, DDIT3 (CHOP), GADD45A, GML, GTSE1, HUS1, MAP2K6, MAPK12, PCBP4, PPP1R15A, RAD17, RAD9A, SESN1, ZAK
Cell Cycle Checkpoint	ATR, BRCA1, FANCG, NBN (NBS1), RAD1, RBBP8, SMC1A (SMC1L1), TP53
Damaged DNA Binding	ANKRD17, BRCA1, DDB1, DMC1, ERCC1, FANCG, FEN1, MPG, MSH2, MSH3, N4BP2, NBN (NBS1), OGG1, PMS2L3 (PMS2L9), PNKP, RAD1, RAD18, RAD51, RAD51L1, REV1 (REV1L), SEMA4A, XPA, XPC, XRCC1, XRCC2, XRCC3
Base-Excision Repair	APEX1, MBD4, MPG, MUTYH, NTHL1, OGG1, UNG
Double-Strand Break Repair	CIB1, FEN1, XRCC6 (G2P1), XRCC6BP1 (KUB3), MRE11A, NBN (NBS1), PRKDC, RAD21, RAD50
Mismatch Repair	ABL1, ANKRD17, EXO1, MLH1, MLH3, MSH2, MSH3, MUTYH, N4BP2, PMS1, PMS2, PMS2L3 (PMS2L9), TP73, TREX1
Other Genes Related to DNA Repair	APEX2, ATM, ATRX, BTG2, CCNH, CDK7, CRY1, ERCC2 (XPD), GTF2H1, GTF2H2, IGHMBP2, LIG1, MNAT1, PCNA, RPA1, SUMO1
Mitochondria	
Membrane Polarization & Potential	BAK1, BCL2, BCL2L1, BNIP3, SOD1, TP53, UCPI, UCP2, UCP3
Mitochondrial Transport	AIP, BAK1, BCL2, BCL2L1, BNIP3, CPT1B, CPT2, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSP90AA1, HSPD1, IMMP2L, MFN2, MIPEP, MTX2, STARD3, TP53, TSPO, UCPI, UCP2, UCP3
Small Molecule Transport	SLC25A1, SLC25A10, SLC25A12, SLC25A13, SLC25A14, SLC25A15, SLC25A16, SLC25A17, SLC25A19, SLC25A2, SLC25A20, SLC25A21, SLC25A22, SLC25A23, SLC25A24, SLC25A25, SLC25A27, SLC25A3, SLC25A30, SLC25A31, SLC25A37, SLC25A4, SLC25A5
Targeting Proteins to Mitochondria	AIP, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSPD1, IMMP2L, MFN2, MIPEP, TSPO
Mitochondrial Protein Import	AIP, COX10, COX18, DNAJC19, FXC1 (TIMM10B), GRPEL1, HSPD1, MIPEP, SH3GLB1

(Continued)

TABLE 13.2 Continued

Categorical Pathways Canonical Pathway (ontology)	Gene Name (Symbol)
Outer Membrane Translocation	TOMM20, TOMM22, TOMM34, TOMM40, TOMM40L, TOMM70A
Inner Membrane Translocation	FXC1 (TIMM10B), IMMP1L, IMMP2L, OPA1, TAZ, TIMM10, TIMM17A, TIMM17B, TIMM22, TIMM23, TIMM44, TIMM50, TIMM8A, TIMM8B, TIMM9
Mitochondrial Fission & Fusion	COX10, COX18, FIS1, MFN1, MFN2, OPA1
Mitochondrial Localization	DNM1L, LRPPRC, MFN2, MSTO1, NEFL, OPA1, RHOT1, RHOT2, UXT
Apoptotic Genes	AIFM2, BAK1, BBC3, BCL2, BCL2L1, BID, BNIP3, CDKN2A, DNML, PMAIP1, SFN, SH3GLB1, SOD2, TP53
Hypoxia Signaling	
Response to Hypoxia	ANGPTL4, ARNT2, CREBBP, EP300, HIF1A, MT3, PRKAA1
Response to Oxidative Stress	CAT, CYGB, GPX1, PIP3-E
Immune Response	GPI, IL1A, IL6, IL6ST, NOS2A, NOTCH1, PTX3, RARA
Other Genes Related to Stress Response	ADM, EPO, HYOU1, VEGFA
Hemoglobin Complex-Associated Genes	CYGB, EPO, HBB, HMOX1, NOS2A, PIP3-E
Peroxidase	CAT, CYGB, GPX1, PIP3-E
Other Oxidoreductase-Related Genes	HIF1AN, HMOX1, MT3, NOS2A, PLOD3, TH
Transcription Cofactors	CREBBP, DRI1, ENO1, EP300, EPAS1, HTATIP, RARA
Transcription Factors	ARNT2, BHLHB2, CREBBP, ENO1, EP300, EPAS1, HIF1A, HIF3A, KHSRP, MYBL2, PPARA, RARA
Other Transcription Factors and Regulators	HIF1AN, NOTCH1
Antiapoptosis	BAX, ANGPTL4, BIRC5, IL1A, MYBL2, PEA15, PRKAA1, VEGFA
Caspase Activity	BIRC5, CASP1
Induction of Apoptosis	BAX, DAPK3, NUDT2
Other Apoptosis Genes	EP300
Signal Transduction	ADM, ARNT2, CASP1, CDC42, CREBBP, EP300, EPAS1, EPO, GNA11, HIF1A, HIF3A, HMOX1, IGFBP1, IL1A, IL6, IL6ST, IQGAP1, KIT, LEP, PLAU, RARA, VEGFA
Protein Biosynthesis	EEF1A1, PDIA2 (PDIP), PRKAA1, RPL28, RPL32, RPS2, RPS7
Protein Heterodimerization	ARNT2, HIF1A, RARA, SAE1
Protein Homodimerization	ARNT2, RARA, VEGFA
Protein Amino Acid Phosphorylation	DAPK3, KIT, PRKAA1
Protein Binding	CASP1, CREBBP, ENO1, EP300, IQGAP1, NOS2A, PEA15, PPP2CB, RARA
Other Genes Related to Protein Metabolism	ARD1A, CDC42, GNA11, HYOU1, MAN2B1, PLOD3, PSMB3, SUMO2, TUBA4A (TUBA1)
Protease Inhibitors	BIRC5, CSTB
Protease Molecules	AGTPBP1, CASP1, ECE1, PLAU, PSMB3
Other Extracellular Molecules	ADM, ANGPTL4, CHGA, COL1A1, EPO, IGF2, IGFBP1, IL1A, IL6, LEP, NPY, PTX3, VEGFA
Cytoskeleton	DCTN2, SPTBN1
Cell Cycle	BAX, BIRC5, EP300, HK2, IGF2, IL1A, MYBL2, SSSCA1, VEGFA
Cell Proliferation	DCTN2, IGF2, IL1A, IL6, MT3, NPY, RARA, VEGFA
Growth Factors	GPI, IGF2, IGFBP1, IL1A, IL6, KIT, VEGFA
Other Genes Related to Cell Growth	ENO1

TABLE 13.2 Continued

Categorical Pathways	Gene Name (Symbol)
Canonical Pathway (ontology)	
JNK Signaling Pathway	EDA2R, TNFRSF19, CD27 (TNFRSF7), MAP2K4, MAPK8, PAK1
Other TNF Superfamily Members and ligands	LTB, PGLYRP1, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF4, TNFSF5IP1, TNFRSF11A, TNFRSF13B, TNFRSF13C, TNFRSF17, TNFRSF19L, TNFRSF4, TNFRSF8
Transcription Regulators	JUN, PARP1, RBL, TNF, TNFRSF1A, TNFRSF25, CD27 (TNFRSF7), TNFRSF9
TNFR1 Signaling Pathway	ARHGDIB, CAD, HRB, LMNA, LMNB1, LMNB2, MADD, MAP3K1, MAP3K7, PAK2, PRKDC, SPTAN1
FAS signaling pathway	IKBKG, LTA, TRAF3, TNFRSF14, TNFRSF1A, TNFRSF1B
Induction of Apoptosis	NFKB1, TNFAIP3
Anti-apoptosis Genes	NFKBIA, TNFRSF1B, TRAF1, TRAF2
Other Apoptosis Genes	NFKB1
Inflammatory Response	CHUK, IKBKB, IKBKG, NFKBIA, TNFAIP3
NF- κ B Signaling Pathway	IKBKB, IKBKG, NFKB1, NFKBIA
Transcription Regulators	DUSP1, HRB, IKBKAP, MAP3K1, MAP3K14, TANK
TNFR2 Signaling Pathway	

role as negative regulators, and lead to the reduction in the activities of these two pathways. Furthermore, p53 transcriptionally regulates TSAP6, Chmp4C, Caveolin-1, and DRAM, which are critical genes in the endosomal compartment, increases exosome secretion and the rate of endosomal removal of growth factor receptors from cell surface, and enhances autophagy [60–63]. It is thought that these p53-mediated activities slow down cell growth and division, conserve and recycle cellular resources, communicate with adjacent cells and dendritic cells of the immune system, and inform other tissues of the stress signals [55, 64, 65].

TNF ligand-receptor signaling occurs because TNF, as a multifunctional cytokine, can induce cell death through receptor-mediated caspase activation and mitochondrial dysfunction by a trigger of oxidative stress induced in cardiovascular disease, neuronal disease, and cancer [66]. Opposing these cell death-promoting signals, binding of TNF receptors can also trigger survival signal activation. A critical balance among various intracellular signaling pathways determines the predominant *in vivo* bioactivity of TNF, as best exemplified by the differential responses of various organs.

A major source of ROS in cells is the mitochondria. Electron leakage from the mitochondrial respiratory chain can react with molecular oxygen, resulting in the formation of the superoxide anion radical, which can subsequently be converted to other ROS. In phagocytes and some cancer cells, ROS can be produced through a reaction that is catalyzed by NADPH oxidase complexes. When attackers from the outside, such as environmental stressors, damage mitochondria, electron leakage is also induced; this dysfunction induces severe problems in tissues [67–70]. Mitochondrial dysfunction causes the onset of some diseases [71–74]. Recent evidence has shown that mitochondrial dysfunction is related closely to insulin resistance and metabolic syndrome. The underlying mechanism of mitochondrial dysfunction is very complex, including genetic factors from both the nucleus and mitochondrial genome, with numerous environmental factors also impacting [75].

Exposure to air pollution, including particles, metals, and other organic compounds as environmental stressors, is associated with pulmonary diseases and cancer. The mechanisms of induced health effects are believed to involve oxidative stress. Oxidative stress mediated by airborne particles and/or fibers might arise from direct generation of ROS from the surfaces of particles and fibers, soluble compounds such as transition metals or organic compounds, and activation of inflammatory cells capable of generating ROS and RNS. Generation of ROS/RNS can cause covalent modifications to DNA directly, or they can initiate the formation of genotoxic lipid hydroperoxides. The resulting oxidative DNA damage can engender

changed gene expression such as upregulation of tumor promoters and downregulation of tumor suppressor genes; the DNA damage might therefore be implicated in cancer development. This chapter describes the important role of free radicals in particle- and fiber-induced cellular damage, the interaction of ROS with target molecules, especially with DNA, and the modulation of specific genes and transcription factor caused by oxidative stress. Consequently, various environmental stressors cause cellular damage through oxidative stress induction and many signaling pathways. However, what environmental stressor is dominant in which signaling pathway is not always clear. Therefore, identifying gene expression signatures extracted from microarray data can clarify how environmental stressors may damage cells and engender diseases.

13.2.1 Case Studies in Tissues or Organs from Rats Exposed to Environmental Stressors

Many animal models have been studied to elucidate mechanisms of action of oxidants or antioxidants. Research on oxidative stress and its defense has expanded dramatically because of its potential benefit in disease prevention and health promotion. In particular, rats exposed to stress-induced chemicals have been extensively studied in biological systems such as cell cultures, animal models, and clinical trials [76–79]. Therefore, 33 independent studies in rats are focused on in this chapter because these studies used microarrays for which gene expression data are publicly available from the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/gds>). Those microarray data with the same platform GPL341 (Affymetrix) sets in rats were downloaded for this study. All data sets were normalized across all arrays by Z-score transformation methods after combination with respect to probe IDs. The normalized values were filtered with oxidative-related genes listed in this work (Table 13.2), and then the top 10 genes from the upregulated and downregulated genes were chosen to analyze gene expression signatures (Table 13.3). The selected genes were classified by using principal component analysis to create gene expression signatures of oxidative stress and were divided into six groups. Most selected genes could be assigned to gene ontology (GO) categories: DNA repair, oxygen and reactive oxygen species metabolism, and response to stress, but cyclins and cyclin dependent kinase contained in “Apoptosis related genes, Cell Cycle Arrest and Checkpoint, Regulation of the Cell Cycle, Regulation of Cell Proliferation, Cell Growth and Differentiation” of “p53 signaling” and “TGF-beta signaling” were not observed. Experimental conditions selected from GPL341 data sets in this work were almost all of short-period exposure using *in vivo* and *in vitro* culture systems of rats. It is noteworthy that

TABLE 13.3 Environmental stressors induce different gene expression signatures

Environmental Stressors (target organ or tissues)	Up-gene	Down-gene	GEO ID
Cluster 1			
Methylprednisolone (kidney)	Apoe, Gpx2, Ngb, Nos2, Prdx6, Tmod1, Tnpl, Tpo	Brcal, Cry2, Fen1, Hus1, Ptgs2, Pttgl, Rad50, Srxn1, Xrcc6	GDS964
Methylprednisolone (liver)	Aass, Atrx, Ncf1, Nqo1, Scd1, Slc41a3, Srd5a2, Tmod1, Tnpl	Chek1, Cry2, Lig1, Mgmt, Pold1, Pold3, Rad50, Rad52, Smc3, Xrcc6	GDS972
Streptozotocin (penile cavernosal)	Apc, Cat, Duox2, Gpx2, Gpx6, Gsr, Lpo, Slc38a1, Smc3, Tpo	Atrx, Gpx7, Nos2, Park7, Ptgs2, Scd1, Slc38a4, Slc41a3, Srxn1, Zmynd17	GDS1393
Trimethyltin (hippocampus)	Apex1, Dnm2, Fance, Gpx7, Lpo, Mgmt, Park7, Prnp, Txnip, Ucp3	Apc, Apoe, Hbz, Mpp4, Ptgs2, Smc3, Srd5a2, Tnpl, Tpo	GDS2555
Octreotide (gastric ECL)	Brcal, Brcal, Dnm2, Duox2, Msh2, Nox4, Tmod1, Tpo, Xirp1	Apex1, Atrx, Cry2, Gpx6, Nos2, Slc38a1, Slc38a4, Slk, Tmod1, Tpo	GDS2558
Cluster 2			
Fibronectin (ventricular myocytes)	Apoe, Atrx, Chaf1a, Ngb, Rad51c, Smc3, Srxn1, Tpo, Zmynd17	Actb, Atrx, Gsr, Mutyh, Ngb, Prdx6, Rad52, Smc3, Tpo, Txnrd1	GDS696
Protein restriction (visceral adipose tissue)	Aass, Apc, Gpx6, Gstk1, Ngb, Prnp, Rad51c, Scd1, Tmod1, Tnpl	Brcal, Chaf1a, Lpo, Mutyh, Nos2, Pttgl, Slc38a1, Slc38a4, Tpo, Ung	GDS880
Heregulin (ureteric buds)	Dher24, Hus1, Ldha, Mif, Park7, Rad1, Rad50, Scd1, Tdg, Ung	Actb, Atrx, Nos2, Nox4, Nqo1, Ptgs1, Rad23a, Srxn1, Txnrd1	GDS1518
Kainic acid (hippocampi)	Apoe, Brcal, Ncf1, Nox4, Pold1, Rad23a, Rad50, Rad51c, Srd5a2, Tmod1	Chaf1a, Hbz, Lpo, Mb, Pold3, Tnpl, Tpo, Ucp3, Ung, Zmynd17	GDS1626
Ethanol (pancreas)	Apoe, Atrx, Hbz, Ogg1, Ptgs2, Scd1, Srxn1, Tmod1, Txnrd2, Zmynd17	Cry2, Hus1, Mb, Msh2, Nox4, Nthl1, Prdx6, Rad52, Slk, Srd5a2	GDS2107
Sulfur dioxide (lung)	Aass, Brcal, Cry2, Hus1, Nos2, Ptgs2, Pttgl, Rad50, Tpo, Zmynd17	Apex1, Brcal, Gpx6, Nos2, Nox4, Rad23a, Rad51c, Srd5a2, Tnpl, Tpo	GDS2372
Hypoxia (adrenal gland)	Chaf1a, Duox2, Ldha, Ngb, Pold3, Rad23a, Slc41a3, Tpo, Txnrd2	Aass, Apc, Apoe, Atrx, Cry2, Lpo, Nox4, Rad52, Srd5a2, Tnpl	GDS2457
Methylprednisolone (skeletal muscles)	Aass, Atrx, Hbz, Ngb, Rad1, Scd1, Slc38a5, Tmod1, Tpo, Xirp1	Als2, Atrx, Brcal, Cat, Gsr, Ncf1, Nox4, Nqo1, Slc41a3, Trpc2	GDS2688
Cluster 3			
Forskolin (pheochromocytoma cell)	Aass, Apex1, Brcal, Chek1, Duox2, Gpx2, Hbz, Nxn, Ptgs1, Pttgl	Atrx, Cat, Cygb, Ehd2, Gpx3, Gpx4, Gpx7, Scd1, Sod3, Vim	GDS1363
N-methyl-N-nitrosourea (mammary tumors)	Cat, Ehd2, Gadd45a, Gstk1, Mgmt, Prdx3, Prdx6, Scd1, Srxn1, Ube2a	Dpagt1, Gab1, Gpx3, Lpo, Mpg, Nxn, Prdx4, Prnp, Rad52, Txnip	GDS1452
Retinoic X receptor ligand LG100268 (mammary gland)	Brcal, Dnm2, Gpx6, Hbz, Mpp4, Ncf1, Nos2, Slc38a1, Tpo	Aass, Atrx, Chaf1a, Gsr, Idh1, Nox4, Prdx1, Rad23a, Xrcc1, Zmynd17	GDS1922
Angiopoietin-1 (aortic rings)	Apex1, Dnm2, Mgmt, Ngb, Pold3, Rad50, Slc38a1, Srd5a2, Srxn1, Ucp3	Atrx, Brcal, Chaf1a, Gpx6, Mb, Nox4, Rad23a, Slk, Tpo, Zmynd17	GDS2037

Isoflurane (basolateral amygdalae)	Brca2, Gpx2, Ifi172, Mif, Nos2, Ptgl1, Rad1, Rad51c, Tpo, Ung	Atrx, Atrx, Gsr, Nox4, Pold3, Prnp, Ptgs2, Scd1, Smc3, Xrcc6	GDS2073
Fe deficiency (jejunum)	Aass, Gadd45a, Gsr, Nqo1, Srxn1, Tdg, Tmod1, Txnrd1, Xrcc1,	Gpx7, Hba-a2, Lpo, Mgmt, Nthl1, Pms2, Rad52, Smc3, Xpc, Xrcc6	GDS2093
Pregnenolone16alpha-carbonitrile (liver)	Dnm2, Gpx6, Lpo, Nqo1, Prdx5, Ptgs2, Scd1, Srxn1, Tpo, Txnrd1	Aass, Als2, Apoe, Hbz, Nos2, Rad51c, Slc38a5, Srd5a2, Tpo	GDS2194
Particulate matter (TPM)/1 of cigarette smoke (lung)	Aass, Apc, Brca1, Brca2, Cry2, Gpx2, Hus1, Slc38a4, Tpo, Txnrd1	Chaf1a, Mb, Mutyh, Nos2, Pold3, Ptgs2, Rad50, Tmod1, Tnp1, Tpo	GDS2616
Genistein (mammary epithelial cells)	Atrx, Brca2, Hba-a2, Ngb, Rad23a, Rad52, Smc3, Tpo, Ung, Zmynd17	Apex1, Brca1, Gpx6, Lpo, Ptgl1, Slc38a4, Srd5a2, Tnp1, Tpo	GDS2639
Aging (hippocampi)	Atrx, Ehd2, Gadd45a, Gtf2h1, Mgmt, Ncf1, Nthl1, Ptgs2, Ptgl1, Srxn1	Ercc6, Mlh1, Pms2, Rad50, Rad52, Slc38a1, Trpc2, Txnip, Wrnpl, Xpc	GDS2774
Depolarization. (midbrain)	Apc, Apoe, Atrx, Brca1, Pold3, Ptgs2, Rad23a Slc38a4, Smc3, Zmynd17	Apex1, Atrx, Chaf1a, Gpx2, Hba-a2, Nos2, Ptgl1, Srxn1, Tmod1, Tnp1	GDS2901
Aristolochic acid (kidney)	Apoe, Atrx, Cry2, Ngb, Ppp1r15b, Scd1, Srxn1, Tpo	Apoe, Atrx, Fen1, Gadd45a, Gpx6, Ifi172, Pold3, Rad52, Txnip, Zmynd17	GSM1038
Cluster 4			
Pyridine activator (ventricular myocytes)	Aass, Chaf1a, Dhcr24, Nthl1, Pinx1, Pold3, Rad52, Scd1, Slc38a1, Xirpl	Apex1, Brca2, Cry2, Gpx6, Hus1, Lpo, Mutyh, Pold1, Rad51c, Tpo	GDS902
Reinnervation (tibialis anterior muscles)	Apex1, Atrx, Chk1, Gpx6, Mgmt, Ncf1, Nox4, Pold3, Smc3, Tnp1	Atrx, Brca1, Chaf1a, Lpo, Nthl1, Rad50, Slc41a3, Txnrd2, Ung, Zmynd17	GDS2243
Hyperinsulinemia (kidney)	Apoe, Chaf1a, Gpx6, Hbaa2, Lpo, Ngb, Ptgs2, Scd1, Slk, Srd5a2	Apc, Atrx, Duox2, Hbz, Mb, Ncf1, Slc38a4, Tmod1, Tnp1, Txnip	GDS2361
Cluster 5			
Sulfur mustard bis-(2-chloroethyl) sulfide (lung)	Apoe, Gadd45a, Gpx2, Hba-a2, Mif, Prdx5, Ptgs2, Scd1, Smc3, Srxn1	Apc, Atrx, Dnm2, Duox2, Gab1, Gpx6, Mutyh, Nox4, Srd5a2, Tpo	GDS1027
Amoxicillin (intestine)	Apc, Apoe, Atrx, Lpo, Mutyh, Slc38a4, Tnp1, Tpo	Apex1, Chaf1a, Cry2, Gpx2, Ngb, Nox4, Scd1, Tpo, Trpc2, Zmynd17	GDS1273
Ischemia (heart)	Apc, Apoe, Gpx7, Nos2, Nox4, Nxn, Prdx4, Rad52, Scd1, Smc3	Atrx, Brca1, Chaf1a, Hus1, Lpo, Pold1, Prdx5, Rad51c, Slc38a4, Xirpl	GDS1959
Cluster 6			
Carbon tetrachloride (liver)	Chaf1a, Ehd2, Gpx2, Hba-a2, Ncf1, Prnp, Ptgs2, Slc38a4, Vim, Zmynd17	Apoe, Dpagt1, Gab1, Hus1, Nos2, Nxn, Ptgs1, Slk, Trpc2, Txnip	GDS1354
Dexamethasone (marrow-derived stromal cells)	Apoe, Ehd2, Gpx6, Mgmt, Mpp4, Srd5a2, Tmod1, Tpo	Apex1, Apoe, Chaf1a, Dnm2, Nos2, Rad50, Rad51c, Slk, Smc1a, Smc3	GDS2231

microarrays capture only transient responses to oxidative stimuli. However, we can predict the underlying mechanism of environmental stressors through oxidative signatures for gene expression. For example, methylprednisolone [80, 81], streptozotocin [82], trimethyltin [83], and octreotide [84] upregulate GPXs, NOS, and NOX, suggesting that environmental stressors in cluster 1 can activate the NO signaling that leads inflammation or other cellular damage. Thioredoxin interacting protein, Txnip, was identified as a unique gene in this category. In cluster 2 (GDS696 [85], GDS880 [86], GDS1518 [87], GDS1626 [88], GDS2107 [89], GDS2372 [90], GDS2457 [91], GDS2688 [92]), Rad23, Rad50, and Rad51c, which are DNA repair and recombination proteins, and the other DNA replication proteins DNA-directed DNA polymerase delta (Pold1) and Pold3 were classified. This classification suggests that environmental stressors in cluster 2 such as fibronectin, protein restriction, heregulin, kainic acid, hypoxia, and ethanol harmed mitochondria or damaged DNA more than the stressors in cluster 1. In cluster 3 (GDS1363 [93], GDS1452 [94], GDS1922 [95], GDS2037 [96], GDS2073 [97], GDS2093 [98], GDS 2194 [99], GDS2616 [100], GDS2639 [101], GDS2774 [102], GDS2901 [103], GDM1038 [104]), Gadd45a, Nthl1, Mgmt, Mpp4, Chek1, Cry2, and Txnrd1 were observed as upregulated genes. Since these genes interact with DNA repair and are p53 signaling activated, it is possible that environmental stressors in cluster 3 cause DNA damage and remodeling. In cluster 4 (GDS902 [105], GDS2243 [106], GDS2361 [107]), DNA replication proteins Pinx1 and Slk were detected as unique genes. In particular, STE20-like kinase (Slk) appears to influence cell survival and proliferation. In fact, Slk has been suggested to have a central growth-suppressive role for Mst orthologs, with intriguing possible links to other established tumor suppressors through work in model organisms. A part of the genes in cluster 5 (GDS1027 [108], GDS1273 [109], GDS1959 [110]) were overlapped in clusters 1 and 3. In cluster 6 (GDS1354 [111], GDS2231 [112]), a part of the genes were overlapped in clusters 2 and 4. However, Vim was detected as a unique gene in GDS1354, which is an experiment in cirrhotic rats [111], since upregulation of this gene was also observed in renal cell carcinoma [113], cerebral tumors [114], and germ cell and trophoblastic neoplasms [115].

13.2.2 Prediction of Biological Influences from Gene Expression Signatures in Rats Exposed to Environmental Stressors

These clusters were characterized by several biological functions. Data of gene expression signatures in Table 13.3 were analyzed through the use of Ingenuity

Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The Functional Analysis identified the biological functions that were most significant to the data set. Molecules from the data set that met the expression value associated with biological functions and/or diseases in Ingenuity's Knowledge Base were considered for the analysis. Right-tailed Fisher's exact test was used to calculate a *P*-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. In Table 13.4, the highest probability predictive function in cluster 1 showed "DNA Replication, Recombination, and Repair"; that in cluster 2 showed "Small Molecule Biochemistry"; that in cluster 3 also showed "Small Molecule Biochemistry"; that in cluster 4 showed "DNA Replication, Recombination, and Repair"; that in cluster 5 showed "Cancer"; and that in cluster 6 showed "Lipid Metabolism." In "Small Molecule Biochemistry," genes related with "degradation or catabolism of hydrogen peroxide" like CAT, GPX3, and GPX4 and peroxidation of lipid were affected in clusters 2, 3 and 6. In "Gene expression," genes related with "binding of p53 consensus binding site" like APEX1, BRCA1, and PTTG1 were affected. For instance, the top-rated network activated by retinoic X receptor ligand LG100268 is shown in Figure 13.1. This network consists of 33 molecules meet 14 molecules, which belong to biological functions of DNA replication, recombination and repair, cancer, and cell cycle.

13.2.3 Oxidative Stress-Mediated p53 Pathways in Human Tissues

Among many oxidative responsive pathways, p53 signaling has been studied extensively and has been thought to play a main role in the orchestration of oxidative events in cells. It coordinates the cellular responses to a broad range of cellular stress factors. In fact, p53 functions as a node for organizing whether the cell responds to various types and levels of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy, as described above in this chapter [37–39]. To control and fine-tune responses to various stress signals encountered by cells, as a transcription factor that both activates and represses a broad range of target genes, p53 demands an exquisitely complicated regulatory network (Fig. 13.2). The classical model for activation of p53 specifically examines three simple and rate-limiting steps: p53 stabilization induced by ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR)-mediated phosphorylation, sequence-specific DNA binding, and target gene activation through interaction with the general transcriptional machinery [29]. Recent studies with animal models describe that mouse double minute

TABLE 13.4 Predicted biological functions by gene expression signatures shown in Table 13.3

Cluster	Predictive Biological Functions	<i>P</i> Value
1	DNA Replication, Recombination, and Repair	1.02E-07
	Hematological Disease	1.33E-06
	Cardiovascular System Development and Function	3.62E-06
	Lipid Metabolism	3.62E-06
	Organ Morphology	3.62E-06
2	Small Molecule Biochemistry	2.34E-07
	Cell Cycle	3.80E-06
	DNA Replication, Recombination, and Repair	3.80E-06
	Cell-to-Cell Signaling and Interaction	3.22E-05
	Cell Death	6.18E-05
3	Small Molecule Biochemistry	2.41E-07
	Gene Expression	7.30E-07
	Cellular Compromise	1.93E-06
	Cell Cycle	4.02E-06
	DNA Replication, Recombination, and Repair	4.57E-06
4	DNA Replication, Recombination, and Repair	7.14E-11
	Cell Cycle	1.18E-05
	Cell Death	2.52E-05
	Respiratory System Development and Function	2.52E-05
	Reproductive System Development and Function	1.10E-04
5	Cancer	8.09E-09
	Gastrointestinal Disease	8.09E-09
	Cell Death	1.20E-07
	Dermatological Diseases and Conditions	5.01E-07
	Organismal Functions	3.57E-06
6	Lipid Metabolism	1.45E-09
	Small Molecule Biochemistry	1.45E-09
	Cell-to-Cell Signaling and Interaction	1.26E-08
	Nervous System Development and Function	1.26E-08
	Cell Death	3.49E-08

(Mdm) 2 and MdmX might determine whether a cell responds to p53 activation with growth arrest or apoptosis, but the molecular mechanism of these differential effects remains unknown. In fact, Mdm2 and MdmX can both be recruited to p53 promoter regions. Via a multitude of mechanisms, they can repress transcription of p53 target genes [116–118]. p53 protein binds sequence-specific regions of DNA of the target gene to process sensing and removal of oxidative damage to nuclear DNA and genetic instability. Furthermore, p53 acts as a transcription factor to regulate the expression of many prooxidant and antioxidant genes. A newly refined model for p53 activation includes three key steps: (1) p53 stabilization, (2) antirepression, and (3) promoter-specific activation. Among the three steps, most environmental stressors contribute mainly to p53 stabilization and promoter-specific activation. Several reports describe that low-weight molecules engender induction of stress-induced genes such as NAD(P)H dehydrogenase, quinone (NQO)1, and NQO2, which stabilize and transiently activate p53 and downstream

genes leading to protection against adverse effects of stressors [119–121].

Therefore, to understand how stress-induced genes are downstream within the p53 pathway, we analyzed gene expression of p53 signaling pathways in array data sets GDS2780 [122] and GSE7967 [123] that had been obtained from the GEO database. In the GDS2780 study, six heavy metals and three organic compounds to which liver carcinoma HepG2 cells were exposed responded dramatically to gene expression of CHK1, CHK2, Cyclin B, Cdc2 p21, p53R2, Cop1-1, and Gadd45 [1]. Interestingly, expression levels of p53R2 and Gadd45 responded differently to the heavy metals: p53R2 is likely to associate with mitochondrial DNA and play a critical role in embryogenesis and neurogenesis [124–128]; in contrast, Gadd45 plays a vital role as a cellular stress sensor in the modulation of cell signal transduction in response to stress. Increasing Gadd45 can stabilize p53 activation, leading to cell cycle arrest or progression to apoptosis [129–131]. Consequently, exposure of cultured human cells to heavy metals

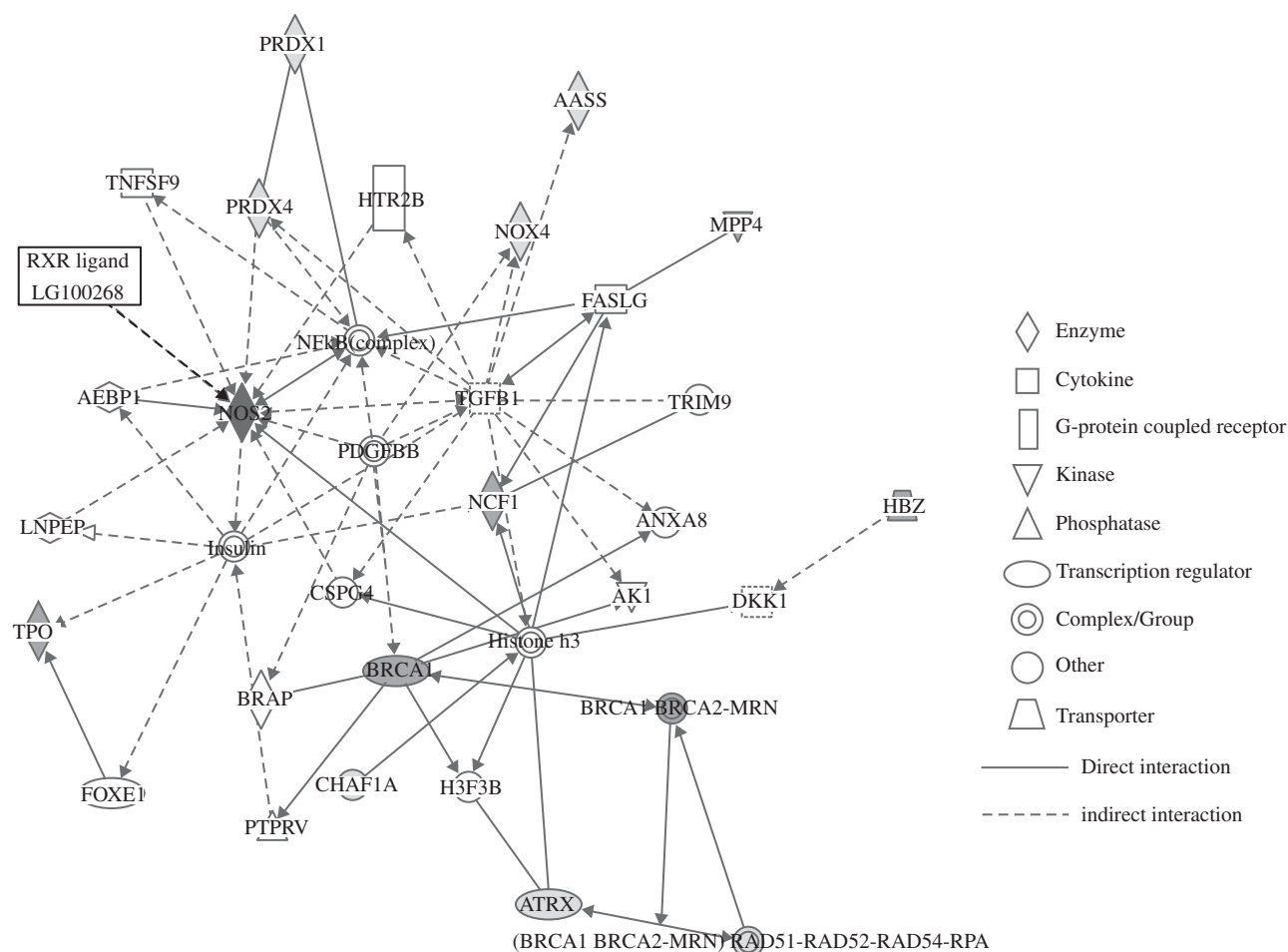


Fig. 13.1 The network generated by retinoic X receptor ligand LG100268 in cluster 3 shown in Tables 13.3 and 13.4: the top-rated network as an example.

dramatically altered the gene expression of oxidative stress-responsive genes. However, in human tissues of the GSE7967 study, the p53 signaling pathway differed from that of heavy metals in the GDS2780 study. Overall, the gene expression signals were weaker than those examined in the GDS2780 study. The GSE7967 study examined cord blood collected at birth from infants whose mothers were exposed or unexposed to arsenic (0.1–68.63 mg/g), showing activation of inflammation and NF- κ B signaling in infants born to mothers exposed to arsenic at high concentration. Therefore, after downloading the data sets, we selected four subjects according to blood concentrations of 0.1, 1.76, 9.66, and 68.63 mg/g; then gene expression of the arsenic exposure-induced responses were visualized in the p53 signaling pathway map. The highest concentration subject showed Gadd45, p53-inducible ribonucleotide reductase small subunit 2 (p53R2), spermatogenic leucine zipper 1 (TSP1), cyclinB, Cdc2, Fas, Noxa, and ATR that were higher than those of the subject with the

low concentration. However, p53 was opposite: high in the low-exposure subject and low in the high-exposure subject, suggesting that the downregulation of p53 facilitates apoptosis and promotes cell proliferation.

Previous works described in our study showed that GSS (glutathione synthetase) and PRDX2 (peroxiredoxin 2) regulated TRADD (TNFRSF1A-associated via death domain), NUDT1 (nucleoside diphosphate linked moiety X-type motif 1), SOD1 (superoxide dismutase 1, soluble), and INSIG1 (Insulin induced gene 1) in the low-exposure group (mean blood concentration 0.142 μ g/g) and that NUDT1 regulated TRADD, TXNRD2 (thioredoxin reductase 2), and PRDX2 in the high-exposure group (21.41 μ g/g), using the theoretical algorithm for identifying optimal gene expression networks (TAO-Gen), which is a Bayesian network algorithm used to describe gene interaction networks [18, 132–134] (Fig. 13.3). In fact, NUDT1 is a DNA repair and recombination protein. The H_2O_2 treatment significantly increased this gene and other oxidative

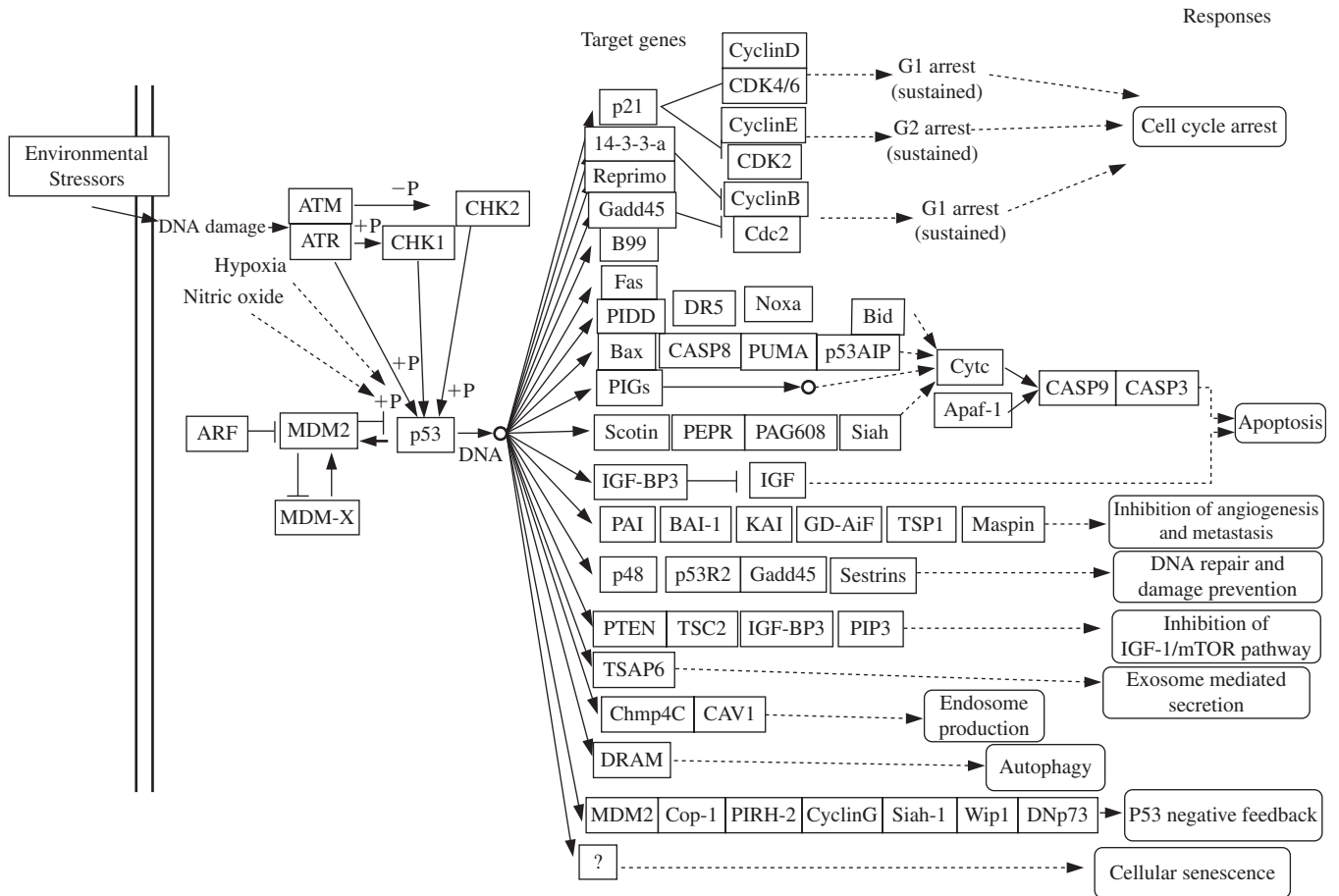


Fig. 13.2 Environmental stressor-mediated p53 signaling pathways. Maps of the p53 signaling pathway partly consulted the KEGG pathway.

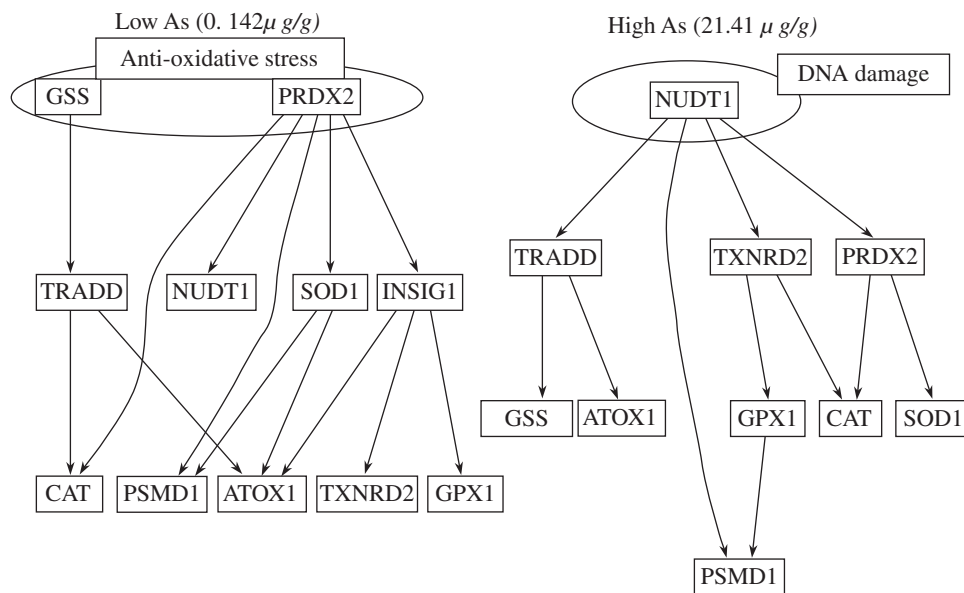


Fig. 13.3 Networks for 11 oxidative stress-related genes selected from the GSE7967 study. TAO-Gen algorithms can predict different mechanisms in low and high exposure to arsenic.

stress genes involved in cell cycle arrest [135]. Results of our analyses suggest that anti-oxidative stress-related genes play key roles in protection against cellular damage in the low-exposure group, but a DNA damage-related gene was dominant in the high-exposure group, in which cell damage would progress. Data sets used in this chapter are from fundamental exposure to environmental stressors in normal tissues and cell lines. Therefore, this discrepancy indicates that gene expression signatures in human clinical tissues or epidemiological studies apparently reflect more inflammation than those of experimental materials, which show acute toxicity in animals after short exposure to oxidants in cell cultures.

13.3 CONCLUSION

In this chapter, we have reviewed gene expression signatures of oxidative stress-mediated signaling pathways by environmental stressors and proposed categorical pathways and canonical pathways of oxidative stress in rat and human systems. Analyses of gene expression signatures in environment-related disease such as neuronal disorders, cancer, and diabetes is an important approach in etiology and risk assessment for human health to elucidate the underlying mechanisms of induced health effects. This will take many more genetic and reverse genetic analyses, combined with functional analysis studies. Furthermore, we have shown that oxidative stress has been associated with many signaling pathways and different environmental stressors impacting different molecules, but they are all connected to the same goals like apoptosis or cell cycle. From a therapeutic point of view, researchers must consider that the best biomarker and/or therapy for oxidative stress-related disease may rely on a combination of several different agents, each specifically targeting one aspect of the oxidative stress machinery.

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SELENOPROTEINS IN CELLULAR REDOX REGULATION AND SIGNALING

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14.1 INTRODUCTION

In 1817 the Swedish chemist Jöns Jacob Berzelius discovered selenium (Se), a chemical element with the atomic number 34 and an atomic weight of 78.96 g/mol. It is a trace nonmetal of the chalcogen group that includes oxygen and sulfur and is present in the Earth's crust and in ocean water at abundances ranging from 0.05 to 2.0 ppm. Sulfur has physical and chemical properties similar to those of Se; however, their noninterchangeable nature in biological systems highlights the substantial differences between these two elements. Se exists naturally as structural analogs of sulfurous molecules, including inorganic selenides (Se^{2-}), selenites (SeO_3^{2-}), and selenates (SeO_4^{2-}), and the organic amino acids selenocysteine and selenomethionine. Although Se is a dietary antioxidant and is required in nanomolar concentrations by organisms in all three domains of life, it is simultaneously toxic, having a median lethal dose in humans of 1.5–6.0 mg/kg body weight with the inorganic salts being more acutely toxic than organic forms [1]. In vertebrates, Se is obtained almost exclusively by ingestion of plants and animals, and the amount of Se in dietary sources depends on the concentration of environmental Se. Currently, the recommended daily allowance of dietary Se for healthy adults is 55 $\mu\text{g}/\text{day}$ and the upper tolerable intake level is 400 $\mu\text{g}/\text{day}$ [2].

Low-molecular-weight selenocompounds are bioactive, but most cellular biological functions of Se are attributed to genetically encoded selenoproteins. Selenoproteins are

defined by incorporation of the 21st amino acid selenocysteine (Sec), which is an analog of cysteine with the sulfur-containing side chain replaced by a Se-containing side chain. Although Se and sulfur have similar chemical and physical properties, key differences generate divergent character between Sec and cysteine, with Sec being a much more efficient catalyst. The cause of the higher catalytic capacity of Sec is uncertain, but a few ideas have been proposed. For example, Sec has been described as having stronger nucleophilic and electrophilic character than cysteine, potentially aiding in catalysis. Additionally, the selenol of Sec has a lower acid dissociation constant than the thiol of cysteine (pK_a of 5.2 vs. 8.3), rendering the selenol a superior leaving group because of its higher acidity. This also means that at physiological pH, Sec tends toward ionization whereas cysteine tends toward protonation, and at low pH Sec retains much higher reactivity than cysteine. The biological rationale for Sec utilization is enigmatic, but rapid recycling of oxidized Sec to the reduced form as well as resisting irreversible oxidation to selenonic acid could potentially explain the biological pressure to use Sec over cysteine in proteins during evolution [3].

Sec is synthesized from serine after aminoacylation to a Sec-specific tRNA (tRNA^{Sec}) that recognizes the UGA “stop” codon, which typically signals to terminate mRNA translation. Recognition of the UGA codon as a Sec insertion site, as opposed to a termination site, requires a Sec insertion sequence (SECIS) element in selenoprotein mRNA molecules, which is a stem-loop structure that is

typically downstream of the UGA codon [4]. Although selenoproteins are present in eukarya, archaea, and bacteria, the mechanism of Sec incorporation differs between domains in several respects. For example, the bacterial SECIS element is located immediately following the Sec UGA codon, whereas the eukaryotic and archaeal SECIS elements are typically located much farther downstream in the 3' untranslated region (UTR). Additionally, the archaeal genus *Methanococcus* has a 5' UTR SECIS element, while eukaryotes have two distinct forms of 3' UTR SECIS elements [5, 6]. An elongation factor specific to Sec-tRNA^{Sec} has been identified as selB in bacteria, whereas the coordinated function of a SECIS binding protein (SBP2) and the eukaryotic Sec-tRNA^{Sec} elongation factor (EFSec) allows the cotranslational incorporation of Sec instead of termination [7]. Biosynthesis of Sec in eukarya and archaea is accomplished by the sequential actions of *O*-phosphoseryl-tRNA kinase (PSTK) and *O*-phosphoserine-tRNA:Sec-tRNA synthase (SEPSECS, SLA), which convert seryl-tRNA^{Sec} to selenocysteinyl-tRNA^{Sec} with a phosphoseryl-tRNA^{Sec} intermediate. Thus Sec formation in archaea and eukarya is a two-step process, whereas bacterial selenocysteine synthase (selA) can synthesize Sec directly from seryl-tRNA^{Sec}. The Se donor utilized by both SEPSECS and selA is selenophosphate, which is generated from selenide (H₂Se) by selenophosphate synthetase (SPS) enzymes. Selenophosphate is first transferred to *O*-phosphoseryl-tRNA^{Sec} by SEPSECS while displacing the phosphoseryl moiety, and subsequent hydrolysis of the phosphate group yields Sec charged to its cognate tRNA [8]. Bacteria have one SPS enzyme termed selD, while two (SPS1, SPS2) have been identified in eukarya, of which SPS2 is itself a selenoprotein. Selenocysteine lyase (SCLY), a putative Se recycling enzyme, is able to catalyze the hydrolysis of Sec into selenide and alanine and potentially delivers Se to SPS enzymes for phosphorylation and subsequent reinsertion into nascent selenoproteins [9]. Pyridoxal phosphate is a prosthetic group derived from vitamin B6 and is required by enzymes involved in metabolism of amino acids, glucose, lipids, and neurotransmitters. The enzymatic activity of both SCLY and SEPSECS is pyridoxal phosphate dependent, implying that Sec synthesis shares regulatory elements with standard metabolic pathways [8, 10, 11].

Several steps in selenoprotein synthesis are regulated by Se availability (dietary or environmental depending on the organism), as well as by the level of oxidative stress. For example, in addition to being a limiting substrate for SPS2, Se levels affect different SECIS elements differentially, potentially regulating selenoprotein synthesis efficiency at the level of translation [7]. Oxidation of the cysteine-rich, redox-sensitive domain of SBP2 masks the nuclear-export signal (NES), causing importation into the nucleus. Subsequently, it is either

sequestered there during high oxidative burden or else reduced by nuclear-specific isoforms of selenoproteins, unmasking the NES for binding and exportation by the nuclear export receptor CRM-1 [12]. As discussed below, individual selenoprotein expression also responds differentially to Se availability and oxidative stress, providing another level of regulatory control for selenoprotein synthesis. Thus there is a complex, nonlinear interaction between Se status and oxidative burden that coordinates the synthesis of the numerous selenoproteins. Despite a low environmental availability of Se and the various elaborate mechanisms for Sec incorporation, selenoproteins are widespread in organisms, with certain plants and fungi being the only major exceptions that lack selenoproteins and an essential biological function for Se [13].

14.2 OXIDATIVE STRESS AND SELENOPROTEINS

Oxidative stress is a broadly used term, but it generally signifies an imbalance in prooxidants and antioxidants in cells and their environment, causing disruption of redox control and macromolecular damage. Here we consider free radicals and nonradical oxidants separately as prooxidants, and distinguish macromolecular damage as a mechanistically different outcome from disrupted redox control. Macromolecular damage to DNA, proteins, and lipids is caused by redox reactions with free radicals, which contain an unpaired electron and are scarce in biological systems because of enzymes and chemicals that rapidly scavenge them and typically generate nonradical oxidants [14]. For example the superoxide radical (O₂^{•−}) is rapidly converted by superoxide dismutase to the nonradical oxidant hydrogen peroxide (H₂O₂). Superoxide can also react with nitric oxide (NO), another free radical, to produce the nonradical oxidant peroxynitrite (ONO₂[−]). Although free radical-mediated macromolecular damage is a common end point observed in aging and disease, the rest of this chapter focuses on the role of selenoproteins in redox systems control and signaling.

Because of such rapid conversion in organisms, free radicals are quantitatively miniscule compared to nonradical two-electron oxidants such as hydrogen peroxide, fatty acid hydroperoxides, disulfides, peroxynitrite, aldehydes, and quinones. In a prototypical redox control system, these nonradical oxidants reversibly react with protein thiols to elicit a redox signal that functions in physiological regulation. Details on the mechanisms of reversible thiol oxidation as a biological control system are beyond the scope of this chapter, but they generally include reaction with an active site cysteine,

modification of a distal allosteric cysteine, and alteration of macromolecular interactions [15]. If the relevant cysteine is in an active site such modifications may serve as a simple binary on-off “switch” to activate or inactivate the protein. Another scenario in which the cysteine residue is not in the active site allows for allosteric regulation, which can throttle protein function along a continuous gradient, akin to a “dimmer.” Finally, oxidation of cysteine thiols is known to control intra- and intermolecular protein interactions by formation of disulfides. Among other things, disulfide cross-linking is well established in maintaining proper protein structure, regulating the viscosity of mucus, and connecting actin filaments and tethering proteins to the cytoskeleton [16, 17].

Selenoproteins are closely linked with the cellular thiol-disulfide couples, particularly the glutathione (GSH) and thioredoxin (TXN) couples. GSH is a tripeptide made of glycine, cysteine, and glutamate and is the most abundant thiol in cells, present at millimolar concentrations. Oxidation of the cysteine thiol links two molecules of GSH to form glutathione disulfide (GSSG). This reaction can be spontaneous in the presence of electrophiles or, alternatively, can be catalyzed by a number of enzymes that utilize GSH as an electron donor, including glutathione peroxidase (GPX), glutaredoxin, and glutathione *S*-transferase enzymes. Reduction of GSSG, producing two molecules of GSH, is performed by the homodimeric flavoenzyme glutathione reductase.

All of the characterized selenoproteins that function as enzymes are oxidoreductases that catalyze thiol-disulfide oxidation-reduction (redox) reactions and contain Sec in the active site. In the early 1970s the first Se-dependent enzyme discovered was glutathione peroxidase 1 (GPX1), which catalyzes the reduction of hydrogen peroxide (H_2O_2) by oxidation of two molecules of GSH to GSSG [18]. Cytosolic and mitochondrial forms of the GPX1 enzyme are transcribed from the same gene containing one Sec-encoding TGA. The enzyme functions as a homotetramer utilizing four Se atoms per active enzyme [19]. GPX1 is the most abundant glutathione peroxidase and the most abundant selenoprotein in rats, representing a significant fraction of the total circulating Se pool [20].

Four homologous GPX selenoproteins have subsequently been identified in humans. GPX2, also known as gastrointestinal GPX, is a cytosolic enzyme that is specific to epithelial cells and is abundant in the gut [21]. The extracellular GPX3 has broad substrate specificity and is found in most extracellular compartments but is abundant in kidney and blood plasma [22]. GPX6 is closely related to GPX3 but is only expressed in the olfactory system and exists as a cysteine homolog in rodents [23]. GPX4 is structurally and functionally different from the other GPX enzymes because it is

active as a monomer rather than a tetramer and can directly reduce membrane lipid hydroperoxides and free fatty acid hydroperoxides. Alternative splicing and transcription initiation generates three distinct isoforms of GPX4 that localize to the cytosol, mitochondria, and nucleus. Additionally, GPX4 translation is regulated by the mRNA binding protein guanine-rich sequence-binding factor 1 [24]. Genetic deletion of GPX4 in mice causes embryonic lethality, and knockdown of GPX4 in cells leads to rapid lipoxygenase-mediated lipid peroxidation and subsequent apoptosis, suggesting that removal of lipid hydroperoxides by GPX4 is essential for cell viability [25, 26]. There are three additional GPX enzymes (GPX5, GPX7, and GPX8) that are not selenoproteins in humans.

TXN is a small protein of ~12 kDa and is present at concentrations several orders of magnitude below GSH. It contains an active site dithiol that is highly conserved in evolution and widely distributed among the TXN superfamily. Through oxidation of the dithiol to a disulfide, TXN can directly reduce cysteine sulfenic acids and control the state of dithiol-disulfide motifs in target proteins, and can also serve as an electron-donating cofactor for enzymes such as ribonucleotide reductase, peroxiredoxins, and methionine sulfoxide reductases [27]. In turn, reducing oxidized TXN is mediated exclusively by the thioredoxin reductase (TXNRD) family of selenoproteins [28]. At least four selenoproteins (GPX3, GPX4, SEPP1, SEPX1) can utilize TXN as a cofactor for enzymatic reduction, and it is possible that others do as well [29, 30]. There are also numerous thioredoxin-like proteins that may depend on TXN or act in parallel to provide additional substrate specificity beyond that provided by TXN. Several selenoproteins contain a TXN-like fold, which is a well-described secondary/tertiary structure pattern with a conserved Cys-X-X-Cys or Cys-X-X-Ser/Thr active-site motif characteristic of oxidoreductases [31], where X is any amino acid. While it is tempting to speculate that these selenoproteins operate similarly to TXN, namely, by controlling the redox state of cysteine residues and dithiol motifs, there is at present little evidence to support or deny this notion.

Three mammalian thioredoxin reductases are selenoenzymes encoded by individual genes. TXNRD1, TXNRD2, and TXNRD3 encode homodimeric flavoproteins that localize to the cytosol, mitochondria, and testes, respectively. They are members of the pyridine nucleotide-disulfide oxidoreductase family and contain two redox-sensitive sites in the N- and C-termini that interact in a head to tail dimer conformation of the active enzyme. These enzymes are capable of reducing a number of substrates, but they depend on NADPH for donating electrons, which are first transferred to the FAD group, then passed to the N-terminal dithiol of one subunit and

subsequently to the C-terminal selenenyl-sulfide of the other subunit. The highly conserved Sec-containing C-terminal motif is absolutely critical for catalytic function of TXNRD enzymes [27, 32]. The main substrate for TXNRDs is the small redox-sensitive protein TXN, which is integral to physiological processes such as cell communication, metabolism, proliferation, and apoptosis. In general, the reactive dithiol of TXN will become oxidized to a disulfide during reduction of an oxidized target protein. Regeneration of reduced TXN proteins requires TXNRD, and thus the TXN/TXNRD system is completely dependent on Se in mammals. The importance of this system is highlighted by the fact that knockout of either TXNRD1 or TXNRD2 is embryonic lethal in mice [33, 34]. It is worth noting that TXNRDs from mammals differ from the Se-independent enzymes of archaea, bacteria, yeast, and plants.

Reactions between target proteins and TXN can be spontaneous, but several enzymes can catalyze the reduction of target proteins using TXN as an electron-donating cofactor. The human genome codes for four methionine sulfoxide reductase (MSR) enzymes that reduce oxidized methionine residues in proteins utilizing TXN as a cofactor. There is now considerable evidence that, like cysteine, reversible methionine oxidation can regulate protein function [35]. For example, calcium/calmodulin-dependent protein kinase II and the phosphatase calcineurin, among many other proteins, are regulated by methionine redox status [36, 37]. A single MSRA and three MSRB enzymes stereospecifically reduce S- and R-sulfoxidated methionines, respectively. MSRB1 is a selenoprotein also known as Selenoprotein R and SEPX1, while MSRA as well as MSRB2 and MSRB3 are Se-independent enzymes. SEPX1 is a zinc-containing protein present in the cytosol and nucleus and exhibits the highest methionine-R-sulfoxide reductase activity because of the presence of Sec in its active site [38]. Interestingly, redox status of the cysteine-rich metallothionein/thionein couple dictates zinc loading in that reduced thionein binds zinc and oxidation of metallothionein releases it. Moreover, thionein can reduce nonselenoprotein MSRB3 in the presence of TXN, TXNRD, and NADPH more efficiently than without thionein [39]. Therefore regulation of specific kinases, phosphatases, and other proteins by methionine-R-sulfoxide reduction is mediated by two selenoproteins (MSRB1, TXNRD1) and NADPH.

14.3 SELENOPROTEINS AND REDOX SYSTEMS

The GSH and TXN couples are central to a global system of connected redox elements involving reversible

oxidation of proteins containing cysteine, methionine, and Sec residues. GPX and TXNRD selenoproteins, being critical effectors of peroxide and thioredoxin reduction, have a direct role in the redox systems biochemistry of organisms (Fig. 14.1A). Oxidative stress in the form of disrupted redox control arises when cysteine (or methionine) residues become abnormally oxidized or irreversibly modified, stripping the reversible redox reactivity that is required for physiological processes. Several covalent and noncovalent modifications to distal or active site cysteine residues modify protein structure and function. A redox-active cysteine residue can become reversibly oxidized to sulfenic acid, which can be reduced stepwise by two molecules of reduced GSH. Additionally, cysteinyl-S-glutathione conjugates are semistable in certain proteins, and S-glutathionylation can lead to altered protein function as observed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and caspase 3 [40, 41]. If two neighboring reduced cysteine residues (dithiol) are reactive, the pair can be reversibly oxidized to a form a cysteine disulfide bridge, as with TXN. The gaseous molecules NO and hydrogen sulfide (H_2S) also react with cysteine residues to produce modified derivatives that are functionally relevant. For example, S-nitrosylation of Cys118 in Ras activates the protein by stimulating guanine nucleotide exchange, while sulphydration, or the addition of a sulfhydryl group from hydrogen sulfide gas to a cysteine thiol, has recently been demonstrated to augment the activity of GAPDH [42, 43]. Proteins often contain multiple cysteine residues that can be reversibly modified (e.g., dithiol-disulfide, S-glutathionylation, S-nitrosylation, S-sulphydration, etc.) to affect protein function and/or interactions.

From a systems perspective, cellular redox status is best understood in terms of redox potential. The redox potential (E_h), or electron motive force, for an oxidation/reduction couple depends on the inherent tendency of the molecule to accept/donate electrons relative to the standard hydrogen electrode and the concentrations of the acceptor and donor (oxidized and reduced species of the couple). In biological systems the redox potential of the major thiol/disulfide couples (GSH and TXN) is maintained at stable nonequilibrium conditions, or in other words, the mean E_h of the GSH and TXN couples in subcellular compartments is nonzero. These redox couples, along with the Cys/CySS (cysteine/cystine) couple and protein Cys residues involved in redox signaling, are in equilibrium neither with each other nor with the NADPH/NADP⁺ couple. In addition to nonequilibrated redox potentials between couples within a subcellular compartment, redox potential is also not equilibrated across subcellular and extracellular compartments. The mitochondria and nucleus tend to be highly reduced (redox potential farther from zero),

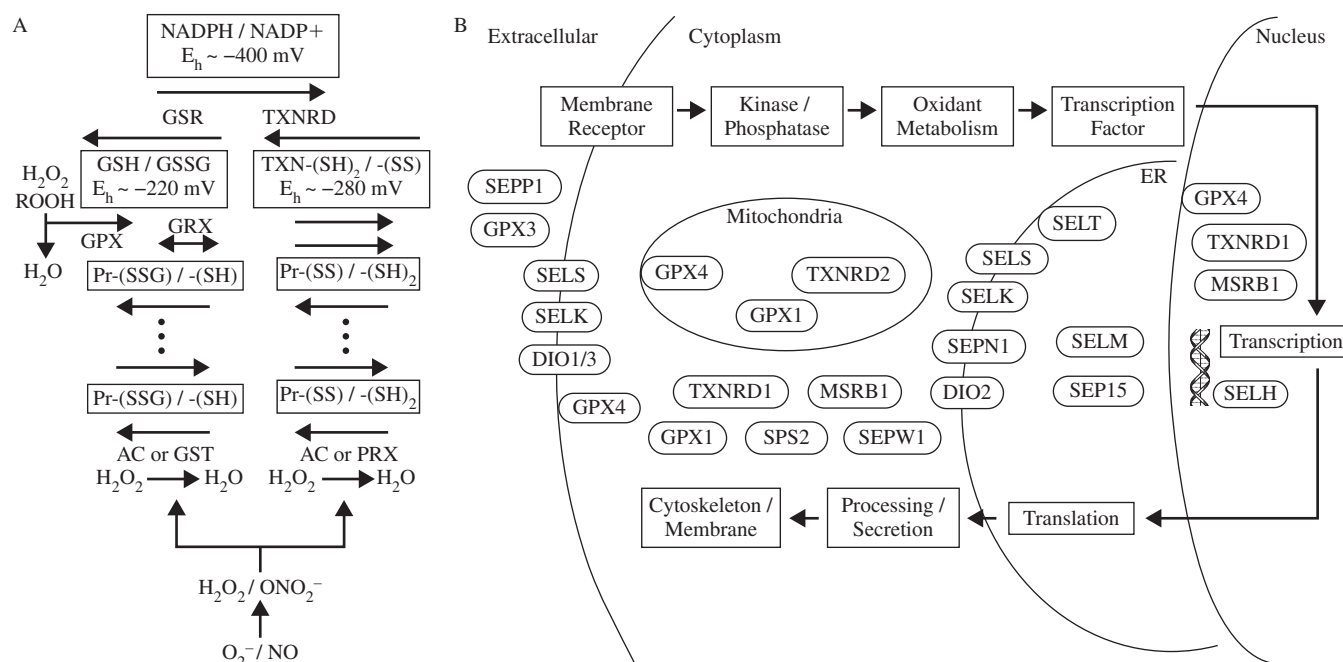


Fig. 14.1 Selenoproteins modulate redox control networks and cellular signaling pathways. (A) Thioredoxin (TXN) and glutathione (GSH) are central hubs of redox control networks. Both electron transport pathways ultimately rely on NADPH as an electron donor. GSH reductase (GSR) and TXN reductase (TXNRD) reduce the GSH and TXN pools, respectively, to maintain a nonequilibrium steady-state redox potential (E_h). E_h values shown are approximate cytoplasmic redox potentials and differ between subcellular compartments. Hypothetical GS-ylation of a cysteine sulfur switch [Pr-(SH)] can be autocatalytic (AC) or catalyzed by GSH-transferase (GST) enzymes. Glutaredoxin (GRX) enzymes can theoretically catalyze GS-ylation or removal of the GSH moiety depending on the local concentration of GSSG and GSH. GPX selenoenzymes oxidize the GSH pool to GSSG in the presence of organic and inorganic peroxides. A similar pathway is drawn for the TXN network sulfur switches, which are depicted here as protein dithiols [Pr-(SH)₂]. Vertebrate TXNRD enzymes are selenoproteins, making the entire TXN network dependent on selenium. The TXN-dependent switches can be autocatalytic (AC) or substrates for catalysts that are oxidized by prooxidants such as peroxiredoxins (PRX) and methionine sulfoxide reductases (not shown). Thus oxidation of a switch is determined by its proximity to the source of oxidant generation or activity of the catalyst. The ellipsis between hypothetical proteins illustrates that series of redox switches can be arranged in parallel circuits that depend on GSH or TXN, and ultimately NADPH. (B) Selenoproteins and redox elements affect regulation of cell signaling. An idealized receptor-mediated signaling cascade involves several steps that are redox sensitive and may be modulated by selenoproteins. Conserved Cys-rich domains of receptors allow for redox regulation, e.g., the EGF and NMDA receptors are known to be regulated by extracellular redox-dependent mechanisms [115, 116]. An array of kinases and phosphatases are redox sensitive and fine-tune intracellular signaling responses [117]. Downstream generation and clearance of oxidants within localized domains (redox-active endosome, mitochondrion, etc.) can differentially propagate and terminate signaling cascades [118]. Translocation of transcription factors and transcriptional activation are under GSH- and TXN-mediated control, while TXN interacts with TXN-like domains of elongation initiation factors to regulate translation [15]. Secretory and transmembrane proteins undergo extensive redox-dependent processing and trafficking in the secretory pathway [119]. Finally, redox-active cysteine residues in receptors, cell adhesion molecules, and cytoskeletal proteins determine receptor function, cytoskeletal structure, and cell surface interactions [120, 121]. Compartmentalization of selenoproteins may provide a functional mechanism for differential control of signaling steps within subcellular regions. Note that six human selenoproteins (GPX2, GPX6, TXNRD3, SELI, SELO, and SELV) have been omitted because of uncertain subcellular and/or restricted tissue distribution.

while the extracellular compartment is relatively oxidized (redox potential closer to zero), with the cytoplasm, endoplasmic reticulum, and lysosomes displaying intermediate values. The steady-state E_h values for thiol-disulfide couples range from -400 mV for NADPH/NADP⁺ to -60 mV for plasma Cys/CySS [44].

With an intracellular reduced GSH concentration of 1 mM, oxidation of just 18 μ M GSH to GSSG will lower

the GSH/GSSG redox potential (ΔE_h) by ~ 60 mV. This relatively shallow redox potential gradient is sufficient to distinguish between proliferating and apoptotic cells, and can theoretically drive a 100-fold change in the dithiol-to-disulfide ratio in proteins with a reactive dithiol motif [45]. Therefore the current through a redox circuit need only be a fraction of the total electron transfer in cells, provided that spatial or catalytic

mechanisms are able to control reaction rates. Under aerobic conditions peroxide and oxygen are always present in cells; therefore coupling of electron transfer to peroxidase (e.g., GPX) or oxidase reactions can provide an additional energetic driving force to maintain function of low-current redox control circuits. Kinetic and spatial insulation of reactive thiol couples allows biosystems to be highly responsive and dynamic, and concurrently provides specificity because noncatalyzed oxidation-reduction and thiol-disulfide exchange reactions are slow in the cellular environment [44].

S-glutathionylated Sec and diselenide bridges have not been described *in vivo*, but transient and stable selenenyl-sulfide linkages are observed in selenoproteins as either active intermediates or structural features, respectively. For example, the reaction mechanisms for GPX and TXNRD enzymes involve a transient mixed selenenyl-sulfide bond, while rat selenoprotein P (SEPP1) has two such linkages that appear to be a structural feature [32, 46]. Transient nitrosylation and sulfhydration of Sec is thermodynamically possible, but its existence is speculative at present. Free Sec is virtually absent from cells, making a selenocysteine/selenocystine couple unlikely, but selenoproteins are present and could be maintained in a nonequilibrium steady state favoring the reduced form. Kinetic (reaction rate and substrate specificity) and spatial (subcellular and tissue distribution) insulation of the selenoproteins has been described; however, whether selenoproteins represent kinetically *limiting* sites of redox control remains an important question. The standard reduction potential (E_0) of selenoproteins is unexplored, and determining whether the mean E_0 of selenoproteins, particularly the TXN-like selenoproteins, is maintained in a nonequilibrium steady state will help determine whether they are central redox control points. Given the known kinetic advantages of the Sec amino acid compared to cysteine in catalysis, as well as the pivotal positions of GPX and TXNRD selenoproteins in respect to redox circuitry (Fig. 14.1A), it seems possible that one or more selenoproteins could be identified as kinetically limiting redox control points that are rapidly disrupted during oxidative stress. Additionally, several selenoproteins have unknown function, but, given the unique reactivity of Sec, they are well suited to transduce oxidant signals by formation of disulfide bonds in downstream targets even in the reducing cellular environment.

14.4 SELENOPROTEINS IN VERTEBRATE SIGNALING

Specific selenoproteins function at the intersection of cellular and organism metabolism by modulating insulin

and thyroid hormone signaling. The iodothyronine deiodinases (DIO) function in activation and deactivation of thyroid hormone and were the second family of enzymes determined to be Sec-containing selenoproteins in the early 1990s [47]. Thyroid hormone metabolism at the level of both production in the thyroid and local hormone activity in the periphery is reliant on the DIO family of selenoenzymes. Most vertebrates have three DIO enzymes that can deiodinate thyroid hormones to control local availability. These integral membrane protein enzymes are thiol-requiring oxidoreductases that remove iodine atoms from the aromatic rings of thyroxine (T_4), triiodothyronine (T_3), and reverse triiodothyronine (rT_3) [48]. DIO1 is a plasma membrane protein found mainly in cells of the liver and kidney, is capable of deiodinating both the inner and outer rings, and produces most of the circulating T_3 . DIO2 is found in the endoplasmic reticulum of cells in several tissues including the thyroid, heart, skeletal muscle, fat, and the central nervous system and selectively removes the outer ring iodine, making it the primary tissue activator of thyroid hormone by converting T_4 to T_3 . DIO3 is also a plasma membrane protein; however, it is mainly found in fetal tissue and the placenta, selectively removes the inner ring iodine, and thus contributes to thyroid hormone inactivation.

A specific role for a selenoprotein in redox regulation of insulin signaling was established when it was found that overexpression of GPX1 causes hyperinsulinemia and insulin resistance in mice [49]. Moreover, genetic deletion of GPX1 promotes glucose tolerance and insulin sensitivity in mice on a high-fat diet by enhancement of insulin-induced PI3K/Akt signaling [50]. Dietary studies in humans have further suggested that supranutritional levels of Se are associated with type 2 diabetes, while animal studies confirm that both excessive dietary Se and GPX1 overexpression lead to hyperinsulinemia and insulin resistance [51–54]. Peroxide-induced oxidation of PTEN and S-glutathionylation of protein tyrosine phosphatase 1B are affected by GPX1 activity, which thereby modulates insulin receptor activation and insulin resistance [55].

Selenoprotein P (SEPP1) is a unique selenoprotein that contains multiple Sec residues and is also implicated in insulin resistance. Diabetic patients display an increase in hepatic SEPP1 mRNA and serum SEPP1 protein, and purified SEPP1 administered to mice is able to induce insulin resistance and glucose intolerance. Furthermore, knockdown or knockout of SEPP1 in mice improves glucose tolerance and insulin sensitivity, and SEPP1 knockout mice are protected against glucose intolerance and insulin resistance even when on an obesity-inducing diet [56]. Since SEPP1 expression can dictate expression of other selenoproteins including GPX1, its effect on insulin resistance may be direct or indirect. Primate and rodent

SEPP1 contains up to 10 Sec residues, while as many as 17 Sec residues are present in zebrafish. SEPP1 is the most abundant selenoprotein in blood and accounts for as much as 65% of plasma Se in rats [57]. Aside from the multiple Sec residues and high Se content, SEPP1 is also distinct in that it is one of only two extracellular selenoproteins (the other being GPX3 mentioned above), and it appears to be a vertebrate adaptation as it has not been found in the genome of *Caenorhabditis elegans* or *Drosophila melanogaster*. SEPP1 is abundantly produced by the liver and secreted into blood; however, local production and secretion in nearly all tissue systems has been described [58]. Bodily transport of Se to extrahepatic tissues, particularly the brain, testes, and kidneys, appears to be facilitated by receptor-mediated uptake of SEPP1 by the low-density lipoprotein receptor family members ApoER2 (LRP8) and Megalin (LRP2) [59]. The abundant Sec residues of SEPP1 are divided into two regions, with the bulk being located in the C-terminal domain that is required for the Se transport function. SEPP1 also contains an N-terminus Cys-X-X-Sec motif and catalyzes the reduction of lipid hydroperoxides in vitro, utilizing TXN as a cofactor [30, 60]. In addition to a Se transport function and peroxidase activity, SEPP1 exhibits pH-dependent heparin binding and heavy metal binding that likely also function in redox-dependent processes.

The examples of GPX1, SEPP1, and the DIO enzymes modulating insulin and thyroid hormone signaling highlight the fact that vertebrate metabolism is tightly integrated with selenoprotein function. This connection between Se and metabolism in multicellular organisms suggests that cell-autonomous regulation of redox systems and signaling may similarly depend on one or more selenoproteins. The regulatory control of cellular redox signaling by Selenoprotein W is discussed next as an example, bearing in mind that several selenoproteins with uncertain functions could similarly have a role in regulating target protein oxidation state.

Selenoprotein W (SEPW1) was purified in the early 1990s but putatively identified much earlier because of its absence in Se-deficient lambs suffering a myopathy called white muscle disease [61]. Mammalian SEPW1 is a highly conserved cytosolic protein of just 87 amino acids, and SEPW1 orthologs are among the most widely distributed selenoproteins in all species including prokaryotes [6, 62]. The expression level of SEPW1 in vertebrates is very sensitive to dietary Se intake as well as the expression level of SEPP1 [63–65]. Abundant SEPW1 expression is observed in muscle, and SEPW1 transcription during myocyte differentiation is maintained by binding of the myogenic transcription factor MyoD to the SEPW1 promoter [66]. A putative metal-response element in the promoter of the SEPW1 gene was probed in vitro with the use of a luciferase reporter fusion construct, and

luciferase specific activity was found to be stimulated by copper and zinc but not cadmium [67]. Although a bona fide enzymatic activity has not been attributed to SEPW1, the presence of a Cys-X-X-Sec motif in a thioredoxin-like fold may indicate thioredoxin-like redox activity [31].

Recently, SEPW1 was shown to pull down and coimmunoprecipitate with the β and γ isoforms of 14-3-3 protein. This interaction was further confirmed by NMR spectroscopy and extended to identify three loops of SEPW1 that interact with 14-3-3 proteins [68]. 14-3-3 β and γ proteins are scaffolding proteins derived from the YWHAB and YWHAG genes, respectively, and bind a diverse assortment of proteins including kinases, phosphatases, and receptors. In this way 14-3-3 proteins coordinate molecular interactions and participate in cell cycle regulation, metabolism, apoptosis, protein trafficking, and gene transcription [69]. A computational study of SEPW1/14-3-3 interaction suggests that a conserved cysteine of 14-3-3 β and γ (Cys191 and Cys195, respectively) can be reversibly oxidized, with SEPW1 acting as a reducing agent [70]. The oxidized cysteine sulfenic acid of 14-3-3 can putatively react with Sec of SEPW1, producing a mixed complex. Subsequently, the formation of an intramolecular selenenyl-sulfide within SEPW1 would result in 14-3-3 being fully reduced. Oxidized SEPW1 can then migrate away and likely be reduced to its parent state by GSH, which is supported by evidence that a SEPW1 cysteine residue conserved from rodents to primates can be S-glutathionylated [71, 72]. Redox regulation of 14-3-3 proteins by SEPW1 could serve several cellular functions, but an intriguing possibility is presented by the in vitro finding that SEPW1 expression is regulated by the cell cycle and knockdown of SEPW1 induces cell cycle arrest [73]. Therefore SEPW1, through redox regulation of 14-3-3 proteins, may coordinate Se availability and oxidative burden with cellular proliferation, differentiation, and death. This redox-regulated functionality may serve as the basis for the association of SEPW1 with myopathies in livestock and multiple myeloma in humans [74].

The example of SEPW1 is meant to highlight the fact that selenoproteins with unknown roles can impact the cellular response to environmental changes, particularly in relation to growth and stress. In the following section a brief summary of what is known of the remaining selenoproteins is presented, with the biological functions described possibly owing to the activity of the selenoproteins in undefined redox circuits.

14.5 THE SELENOPROTEIN FAMILY

The human genome codes for 25 selenoproteins, most of which have been identified recently by bioinformatics

approaches looking for SECIS elements downstream of in-frame UGA codons [23]. The various members display wide subcellular and tissue distribution, and several are known to have multiple transcript variants and protein isoforms. Selenophosphate-synthetase 2 (SPS2) is a eukaryotic selenoprotein that is required for the synthesis of all selenoproteins including itself. Selenophosphate is generated by SPS2 in the presence of selenide and ATP [75]. A related protein called SPS1 contains a cysteine residue in place of Sec, but its involvement in Sec and selenoprotein biosynthesis is uncertain [76]. It has been suggested that SPS2 assimilates selenite, whereas SPS1 recycles Sec in a Se-salvage pathway [77]. Intriguingly SPS2, but not SPS1, is required for selenoprotein synthesis in NIH3T3 mouse fibroblasts [78]. However, an *in vivo* requirement of SPS2 and specificity of SPS1 and SPS2 with different Se substrates have not been reported.

Oxidative stress can trigger dysfunction of organelles including the mitochondria, nucleus, and endoplasmic reticulum. A subset of selenoproteins is observed in mitochondrion to combat against electron leak during oxidative respiration and phosphorylation. Mitochondrion-specific isoforms of GPX1, GPX4, and TXNRD2 regulate peroxide metabolism and oxidative tone within this organelle. The selenoproteins GPX4, TXNRD1, MSRB1, and SELH have been shown to exhibit varying degrees of nuclear localization (Fig. 14.1B). Apart from SELH, the other three selenoproteins are presumably involved in reduction of lipid peroxides, oxidized TXN, and sulfoxidized methionine residues within the nuclear envelope.

SELH is the only DNA-binding selenoprotein described and has a role in regulation of gene expression. Similar to SEPW1, Selenoprotein H (SELH) is a small selenoprotein that is highly expressed during development and is sensitive to dietary Se intake [79, 80]. Like several selenoproteins, it contains a Cys-X-X-Sec sequence within a thioredoxin-like fold, but unlike any other selenoprotein described to date, it is a DNA-binding protein of the AT-hook family. SELH is primarily located in the nucleus and is implicated in redox-sensitive transcription of genes whose products are involved in *de novo* glutathione synthesis and phase II detoxification [81]. Multiple metal-response elements are present in the SELH gene [82], and one group has confirmed *in vivo* that SELH mRNA and protein are upregulated under conditions of elevated copper in mouse liver [83]. Although it is a nuclear protein, mitochondrial biogenesis and function are also linked with SELH. Overexpression of SELH in a transformed neuronal cell line attenuates the UVB-induced increase of p53 protein and caspase-mediated apoptosis [84]. Additionally, SELH overexpression increases mitochondrial size, cytochrome c content, and expression of

mitochondrial biogenesis proteins while boosting respiration [85]. Collectively these findings suggest that SELH is a Se- and metal-regulated selenoprotein that is able to transduce oxidant signals by modulating gene expression in conjunction with other redox-sensitive transcription factors. Further investigation is warranted to determine whether SELH modifies cysteine *S*-glutathionylation or disulfide formation in target proteins such as p53 to regulate gene expression.

The endoplasmic reticulum (ER) regulates the synthesis, folding, and transport of proteins and additionally constitutes the main intracellular store for calcium ions, which are integral in cell signaling. Seven selenoproteins are enriched in the ER (Fig. 14.1B), and some are postulated to have a role in protein folding and ER calcium handling, since oxidative mechanisms within the ER are known to regulate these processes [86]. The ER is a relatively oxidizing environment compared to other intracellular organelles and contains oxidase enzymes to facilitate the formation of disulfide bonds in proteins destined for export. Simultaneously, GSH and TXN system components are transported into the ER, providing both oxidation and reduction mechanisms for dynamic redox regulation associated with protein processing and secretion. Redox state affects calcium homeostasis by modulating ER calcium channels and chaperones, and oxidative stress and ER stress are intimately related in signaling for apoptosis [87]. Similar to the ER, the secretory and endosomal/lysosomal pathways are also more oxidized than other subcellular compartments [88, 89]. Ligand binding to various receptors stimulates endocytosis of redox-active endosomes whose luminal redox activity directs spatiotemporally regulated signaling and prevents nonspecific redox reactions [90, 91]. Therefore redox-mediated processes are vital for secretory and endocytic function, and the ability of selenoproteins to transmit oxidative signals from reactive intermediates to disulfide bonds of target proteins may help to explain the enrichment of selenoproteins in the ER.

Selenoprotein T (SELT) is an ER- and Golgi-localized selenoprotein that is ubiquitously expressed from development through adulthood and shares some sequence similarity with SEPW1 and SELH including the thioredoxin-like fold containing a Cys-X-X-Sec motif [31]. Deficiency of SELT in murine fibroblasts causes an upregulation of SEPW1, in addition to altering cell adhesion and redox regulation [92]. A biological role for SELT in neuroendocrine secretion and calcium mobilization *in vitro* has also been presented. SELT was identified as a target gene of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), and manipulation of SELT expression altered PACAP-induced intracellular Ca^{2+} changes and growth hormone secretion [93].

Two of the more abundant and ubiquitous ER selenoproteins, Selenoprotein M (SELM) and 15-kDa selenoprotein (SEP15), are both 15-kDa proteins that share 31% sequence homology and have Cys-X-X-Sec and Cys-X-Sec redox motifs, respectively. SELM and SEP15 have thioredoxin-like tertiary structure and homology to protein disulfide isomerases that suggest oxidoreductase activity, but direct evidence in support of this notion is lacking [94]. SEP15 associates with UDP-glucose:glycoprotein glucosyltransferase (UGTR), a protein involved in protein conformation quality control, and has been suggested to facilitate proper protein folding [95]. A role for SELM in protein folding has been proposed, and recent evidence suggests that SELM may also be involved in regulating the flux of calcium ions. Overexpression of SELM in a neuronal cell line *in vitro* reduced peroxide-induced calcium influx, whereas knockdown of SELM increased the baseline intracellular calcium concentration [96].

Selenoprotein N (SEPN1) is a large (70 kDa) single-spanning transmembrane protein localized to the ER membrane with two known isoforms generated by alternative splicing of exon 3 [97]. Several congenital muscular dystrophy syndromes such as multiminicore disease, rigid spine muscular dystrophy, and desmin-related myopathy with Mallory body-like inclusions are directly associated with mutations in the SEPN1 gene and have been classified as SEPN1-related myopathies [98]. Interestingly, mutations in SEPN1 leading to congenital fiber type disproportion are associated with insulin resistance [99]. To date, SEPN1 is the only selenoprotein gene in which mutations are directly and causatively linked to human disease. An *in vivo* study using zebrafish determined that SEPN1 associates with ER/SR ryanodine receptors, and that this interaction is necessary for the calcium-induced release of calcium from intracellular stores [100]. Ryanodine receptor channels are homotetramers with several redox-regulated cysteine residues, and SEPN1 contains a Cys-Sec-Gly-Ser motif, suggesting that SEPN1 regulates ryanodine receptor-mediated calcium flux in muscle by redox-dependent signaling. Finally, SEPN1 is integral to the generation and/or maintenance of skeletal muscle satellite cells, which are an adult stem cell population involved in muscle growth and regeneration [101].

Selenoprotein K (SELK) and Selenoprotein S (SELS) are also predominantly ER-localized single-spanning transmembrane proteins; however, they are much smaller than SEPN1 and also show some localization to the plasma membrane [23, 102, 103]. Both are widely expressed in a variety of tissues and have been implicated in the cellular response to ER stress. Specifically, ER stress agents regulated the expression of SELK in HepG2 hepatoma cells, and knockdown of SELK exacerbated

cell death when challenged with ER stress [104]. Genetic deletion of SELK in mice decreases receptor-mediated calcium flux in immune cells, impairs calcium-dependent immune cell function, and increases West Nile virus-induced lethality [105]. An interesting link between metabolism and inflammation is presented in the case of SELS, which was originally identified as a glucose-regulated protein in a rodent model of diabetes [106]. The relationship between SELS and type 2 diabetes was confirmed in humans, and there is evidence that SELS can be secreted from liver and identified in blood sera, where it associates with LDL [107, 108]. SELS is now known to also be regulated by inflammatory cytokines [109], and reciprocally, reduced expression of SELS, due to polymorphisms in the gene promoter, influences the levels of IL-1, TNF- α , and IL-6 [110]. SELS participates in removal of misfolded proteins from the ER lumen [103, 111] and was demonstrated to prevent ER stress and have antiapoptotic function in macrophages and astrocytes [112, 113].

Three selenoproteins remain largely unexplored, with very little published data currently available. The sequences of Selenoprotein I (SELI), Selenoprotein O (SELO), and Selenoprotein V (SELV) were identified in the human genome several years ago; however, almost no information is available on the cellular localizations or physiological functions of these selenoproteins. SELI mRNA is known to be expressed in several tissues, and it is a putative transmembrane protein hypothesized to function in phospholipid biosynthesis based on the presence of a CDP-alcohol phosphatidyltransferase motif that is conserved in phospholipid synthases [23, 114]. SELO is predicted to be a 669-amino acid selenoprotein containing a Cys-X-X-Sec motif, but experimental data demonstrating a redox function are unavailable [23]. SELV appears to be a testes-restricted protein with a predicted thioredoxin-like fold housing a Cys-X-X-Sec motif and also has some sequence homology with SEPW1, SELH, and SELT [23].

14.6 CONCLUSION AND FUTURE PERSPECTIVES

The implications of selenoproteins functioning as part of the circuits mediated by central couples for redox signaling are widespread. These circuits control networks that integrate and coordinate cellular processes through a series of parallel redox elements, and selenoproteins are positioned in key places to drastically affect circuit function. The GPX enzymes rapidly consume peroxide to limit the distance of locally generated oxidant signals, and in doing so impact the redox potential of the GSH/GSSG couple. TXNRD selenoproteins reduce TXN to

maintain the nonequilibrium steady-state redox potential of the couple and thus are the basis of a functionally diverse array of signaling processes mediated by TXN. Reducing sulfoxidized methionines is one such process that is partially realized by another selenoprotein, MSRB1. DIO enzymes and SEPP1 modulate vertebrate metabolism, while SEPW1 and SELH modulate cellular metabolism. At least four ER-localized selenoproteins (SELT, SELM, SEPN1, SELK) are involved in regulating intracellular calcium flux and consequent cellular signaling, and another two (SEP15, SELS) participate in protein folding. SPS2 is a selenoprotein that regulates synthesis of all selenoproteins including itself, while SELI, SELO, and SELV still have unknown functions. The interaction of selenoproteins with thiol-disulfide pools in humans highlights their importance in redox regulation and signaling, particularly under conditions of oxidative stress. Oxidative stress is associated with diverse disease processes, and redox-sensing selenoproteins may provide a mechanistic link between sources of oxidative stress and the fundamental cell signaling that contributes to disease pathology. Specific selenoproteins could optimize target protein function by modifying reversible reactions (i.e., oxidation, nitrosylation, acylation, sulfhydration or metal binding) that affect macromolecular structure, activity, interactions, and trafficking. Elucidation of specific targets and circuits for selenoprotein activity will create new strategies for targeting relevant redox systems that go awry during disease pathology. Almost two hundred years since Berzelius named Se after the Greek moon goddess Selene, this unique trace element is poised to illuminate a world of redox systems biology.

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ANTIOXIDANT THERAPY AND ITS EFFECTIVENESS IN OXIDATIVE STRESS-MEDIATED DISORDERS

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15.1 INTRODUCTION

Antioxidants are chemical compounds that give an electron to free radical molecules and convert them into a harmless configuration. This prevents damage to chain reaction and can involve lipids, proteins, enzymes, carbohydrates, DNA, cell, and nuclear membranes up to the death of the cell. When the body's scavenging ability is not able to deal with free radical species, that is, reactive oxygen species (ROS) and reactive nitrogen species (RNS), they cause oxidative damage in all the body's tissues, leading to disease. The effect of oxidative stress at the cellular level is illustrated in Figure 15.1. This oxidation-induced damage may be prevented by exogenous or endogenous antioxidants. ROS seem to be an important factor involved in endothelial dysfunction, diabetes, atherosclerosis, and ischemia, while RNS have been associated with arthritis, diabetes, degenerative neuronal diseases, cancer, and atherosclerosis. Under physiological conditions, the overproduction of ROS and RNS and their neutralization are prevented by the activity of the endogenous antioxidative defense system (AOS). It includes enzymes like superoxide dismutase, catalase, glutathione peroxidase, and other antioxidant regenerating enzymes such as glutathione reductase, dehydroascorbate reductase, and glucose-6 phosphate dehydrogenase that maintains reduced NADPH, hydrophilic scavengers like urate ascorbate glutathione,

flavonoids, and lipophilic scavengers, like tocopherols, carotenoids, and ubiquinone. Antioxidants are generally supplied in the diet and include polyphenols, lipoic and ascorbic acid, carotenoids, resveratrol, epigallocatechin-3-O-gallate, lycopene, quercetin, genistein, ellagic acid, ubiquinone, and indole-3 carbinole. The properties of these compounds are involved in the physiological redox balance as they can prevent damage to the tissues due to the oxidation typical of all the biological systems and characterized by the production of highly reactive free radicals [1]. There is growing evidence that ROS play a key role in several pathological conditions and in the aging process. No final conclusion about possible therapy protocols based on the administration of antioxidant compounds has been reached yet, but it can be hypothesized that, in the near future, biochemical investigations, able to detect an oxidative imbalance, may become routine tests necessary to restore the antioxidant natural protective barrier, avoiding irreversible damage. An example of the power of antioxidant supplementation to prevent excessive oxidative stress is given by a study conducted in 400 healthy subjects to assess the effect of physical activity combined with antioxidant treatment (330 ml/day of Funciona™). This study shows that, after 10 months, the oxidative stress caused by exercise was prevented by the antioxidant treatment [2]. There is also a lack of agreement concerning the parameters of oxidative stress or antioxidant state in specific

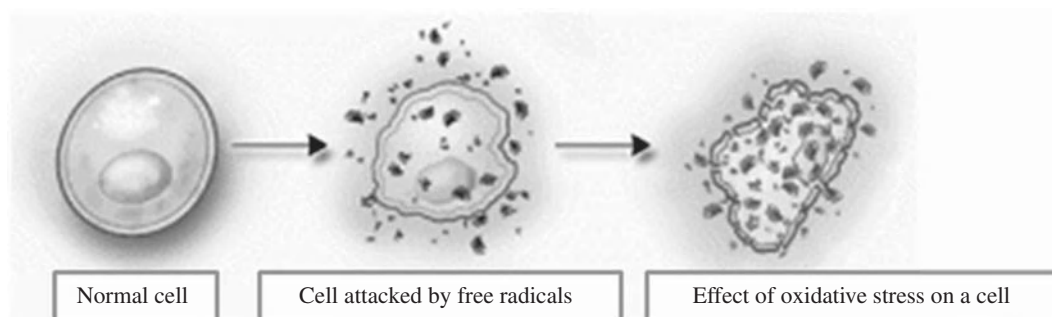


Fig. 15.1 Effect of oxidative stress at cellular level.

patients or diseases, and researchers are trying to find new techniques to monitor oxidative stress in humans. Among other techniques are high-performance liquid chromatography (HPLC) and immunochemical determinations, which have become the common standard. The number of centers using Points of Care is growing among the scientific community because of their low invasiveness, reduced costs, practicality, and ease of use. In particular, Free Oxygen Radicals Testing (FORT) and Free Oxygen Radicals Defence (FORD) have proven their reliability and ease of use [3]. Among the main techniques for free detection is electron spin resonance (ESR), which can provide valuable information on free radicals in solution chemistry, while to examine free radicals in biological materials, spin trapping is the most appropriate technique, which may allow investigators to detect radicals that are normally present at very low concentrations or whose lifetime is too short to be detected by direct ESR analysis. Specific products of DNA oxidation have been proposed as potential general markers of this process. For example Bruce Ames's laboratory, at the Berkeley campus of the University of California (USA), has proposed that thymine glycol and 8-hydroxydeoxyguanosine might be appropriate markers of DNA base oxidation. Analysis of 8-hydroxydeoxyguanosine in extracted DNA is a straightforward method of studying DNA base oxidation in biological tissues, and this substance is also excreted in urine and hence might provide a noninvasive means of assessing whole-body DNA base oxidation. GSH is the major intracellular reductant, and analysis of GSSG:GSH can provide a valid index of cellular oxidative stress. Assays relying on the ability of bleomycin to bind Fe, facilitating the oxidation of DNA (the bleomycin-Fe assay), and of phenanthroline to undertake the same role with Cu (the phenanthroline-Cu assay) have also been developed. Data indicate that small amounts of this "free" or "catalytic" Fe or Cu may be found in biological fluids such as cerebrospinal fluid (CSF) and that these levels increase in some disease states (e.g., Cu or Fe overload).

They provide strong support, as increased free radical production may occur in these situations [4]. An evaluation of 67 randomized trials including participants who were either healthy (primary prevention trials) or affected by different diseases (secondary prevention trials; gastrointestinal, cardiovascular, neurological, ocular, dermatological, rheumatoid, renal, endocrinological, or unspecified) analyzed the effect of several antioxidant supplements, such as beta-carotene, vitamin A, vitamin C, vitamin E, and selenium versus placebo or no intervention, concluding that only vitamin A, beta-carotene, and vitamin E seem to increase mortality [5]. α -Lipoic acid (ALA), also named 1,2-dithiolane-3-pentanoic acid or thioctic acid, is synthesized in humans by the liver, heart, and kidney and has also been widely studied for its antioxidative properties. It is water- and fat soluble and is widely distributed in both cellular membranes and cytosol. A number of experimental and clinical studies point to the usefulness of ALA as a therapeutic agent for several medical conditions, namely, diabetes, atherosclerosis, insulin resistance, neuropathy, neurodegenerative diseases, and ischemia-reperfusion injury [6]. ALA exerts an antioxidant effect in biological systems through direct ROS quenching and via transition metal chelation [7]. Carpal tunnel syndrome (CTS) is the most common peripheral mononeuropathy. One study [8] has compared the efficacy of ALA (600 mg/day) and γ -linolenic acid (GLA; 360 mg/day) to that of a multivitamin B preparation (Vit B6 150 mg, Vit B1 100 mg, Vit B12 500 μ g daily) administered for 90 days in 112 subjects with moderately severe CTS. The following results were observed:

1. A significant reduction in both symptom scores and functional impairment in the ALA/GLA group, while the multivitamin group experienced a slight improvement in symptoms and a deterioration in functional scores.
2. Electromyography showed a statistically significant improvement in the ALA/GLA group, but not in the multivitamin group.

3. ALA/GLA improved symptoms and functional impairment. The improvement was significant in the multivitamin group but less marked than in the ALA/GLA group.

Many compounds have been studied in relationship to their antioxidant properties. Shark liver oil (SLO) contains both alkylglycerols (AKG) and squalene and is an ancient remedy among the fishermen along the west coast of Norway and Sweden. It has been used for wound healing, treatment of irritations of the respiratory and alimentary tracts, and lymphadenopathy. Squalene is the main component of skin surface polyunsaturated lipids as an emollient and antioxidant and has hydration and antitumor activities [9]. SLO supplementation in high doses [3.6 g of squalene, 3.6 g of AKG, and 750 mg of n-3 polyunsaturated fatty acids (PUFA) per day, for 4 weeks] in 13 volunteers showed an increased response of neutrophils toward bacteria, an increased level of C4 component of complement in blood, the rise of the total antioxidant status of serum, and predominance of type 1 cytokine IFN- γ , TNF- α , and IL-2 production by peripheral blood mononuclear cells. The same study has shown that SLO supplementation also markedly affected lipid metabolism and cholesterol balance [10]. Oxidative stress has also been related to lipotoxicity, that is, the process leading to end organ damage and/or dysfunction following excess overload that results not only from unoxidized FAs but also from endogenous lipids synthesized from excess glucose through the process of the *de novo* lipogenesis. It has been demonstrated that this can occur in the presence of excess lipid accumulation in nonadipose tissues such as liver [nonalcoholic fatty liver disease (NAFLD)], pancreas (diabetes), muscle (insulin resistance), and heart (diabetic cardiomyopathy) [11]. An interesting hypothesis has suggested that oxidative stress in obesity may result partly from the accumulation of intracellular triglycerides that, in turn, may elevate superoxide radical generation within the electron transport chain by inhibiting the mitochondrial adenosine nucleotide transporter. This inhibition leads to a diminution in intramitochondrial adenosine diphosphate (ADP) that, in turn, reduces the proton flux through the adenosine triphosphate-synthase reaction (i.e., the adenosine triphosphate-synthase reaction requires ADP as substrate [3]). Another naturally occurring compound, attracting the attention of many researchers and clinicians, is curcumin, a constituent of the spice turmeric and one of the principal ingredients in curry powder. Its active ingredient is diferuloylmethane, a hydrophobic polyphenol with a peculiar yellow color, and it is prepared from the root of the *Curcuma longa*

plant, a member of the ginger family [12]. Curcumin's clinical use has been associated with several pathological conditions because of its antiinflammatory properties, namely, the reduction of NF- κ B, COX2, and proinflammatory cytokines such as IL-1, IL-6, and TNF- α . In particular it seems to improve rheumatoid arthritis, psoriasis, postoperative inflammation, chronic anterior uveitis, and orbital inflammatory pseudotumors [13–18]. Moreover, clinical improvements have been observed in irritable bowel syndrome, tropical pancreatitis (PEP), gall bladder and biliary motility, gastric ulceration, and familial adenomatous polyposis coli [19–25]. It also seems to improve endothelial function in type 2 diabetes mellitus [26] and to lower serum cholesterol [27]. In an open-label study [5 patients with Crohn's disease (CD) and 5 with ulcerative proctitis] curcumin improved clinical and laboratory parameters, with a reduction in need for concomitant medications observed in nine of 10 cases [28]. A larger multicenter randomized, double-blind controlled trial of 89 patients with quiescent ulcerative colitis (UC) showed that two of 43 patients taking curcumin per os had relapsed by 6 months, compared with eight of 39 in the placebo group [29]. A trial involving 25 patients with various different premalignant or high-risk lesions suggested that oral curcumin may have chemopreventive effects in progression of these lesions [30]. In an uncontrolled study of 15 patients with advanced colorectal cancer refractory to standard treatments, 440 mg/day of curcuma extract administered per os showed a 59% reduction in activity of the lymphocytic biomarker glutathione *S*-transferase. Five patients maintained radiologically stable disease over the 2- to 4-month study period [31]. An uncontrolled study involving 62 patients with cancerous oral lesions showed that a topical curcumin application reduced symptoms in 70% of the patients and caused tumor shrinkage in 10% [32]. A study performed in 21 patients with advanced pancreatic cancer treated with high-dose oral curcumin showed a disease stability or regression in four cases [33]. Curcumin is virtually able to affect every stage of carcinogenesis from cell proliferation to angiogenesis and metastasis, and it directly acts on ROS scavenging and production and the NF- κ B/mTOR signaling pathways. This spice seems to be a safe and beneficial agent that may be useful to counteract, even if consumed at low doses, a great number of pathological conditions associated with age [34].

Lately, many experimental studies have investigated berries for their possible beneficial effects on health, namely, prevention of certain types of cancer, cardiovascular diseases, type 2 diabetes, obesity, neurodegenerative diseases associated with aging, and infections. In particular, the research community have focused on red raspberries, which contain a variety of beneficial

compounds including essential minerals, vitamins, FAs, and dietary fiber, as well as a wide range of polyphenolic phytochemicals such as flavonoids, phenolic acids, lignans, and tannins (anthocyanins and ellagitannins) [35].

As to black raspberries, a study [36] showed that freeze-dried blackberries, given to patients diagnosed with Barrett's esophagus, resulted in a significant decrease in oxidative DNA damage. Another study [37] showed, after 6 weeks of treatment consisting of four times daily application of a bioadhesive black raspberry gel on premalignant oral lesions, a significant reduction in COX-II protein levels, suppression of genes associated with RNA processing and growth factor recycling, and inhibition of apoptosis. In the same study a subset of patients displayed posttreatment decrease in lesion microvascular density (MVD). A 4-week intervention [38] with a red berry juice containing red raspberry juice improved the levels of glutathione and reduced DNA oxidative damage in healthy adult men, while when a dessert made from a similar juice was given to elderly subjects for 2 weeks, no effect was observed as to oxidative stress status [39]. Compared with preexercise and control levels, postexercise levels of protein and DNA oxidation in cyclists were significantly decreased in the treatment group receiving an antioxidant-rich beverage containing raspberry, black grape, and red currant concentrates [40]. A 90-day study involving the supplementation of healthy participants with a sea buckthorn berry extract found a significant reduction in C-reactive protein (CRP) level [41], which is associated with inflammation and, in particular, cardiovascular risk.

15.2 AGING

The mitochondria [from the Greek *mitos* ("thread") + *khondrion* ("little granule"), diminutive of *khondros* ("granule, lump of salt")] are oval-shaped, membrane-enclosed intracellular organelles containing their own DNA. They exert several functions, namely, the generation of adenosine triphosphate (ATP) and the regulation of cellular proliferation and apoptosis. They are associated with the aging process since they represent the main intracellular source of ROS, produced by the mitochondrial respiratory chain and the major target of free radical action. ROS may attack mitochondrial proteins, lipids, and mitochondrial DNA (mtDNA), which can lead to mtDNA mutations and, in turn, to impairment of the respiratory chain complexes, increased mitochondrial ROS production, and increasing mitochondrial DNA mutations. Therefore oxidative damage to mtDNA represents a stepping stone for protein mutation, generation of additional free radicals, and altered energy production [42]. From a

chemical perspective, melatonin, together with its metabolites, can act as an endogenous free radical scavenger and broad-spectrum antioxidant. It is characterized by a small size and amphiphilic nature that make it able to reach all the cellular and subcellular compartments. The highest melatonin intracellular concentration seems to be in mitochondria [43]. Melatonin exerts beneficial consequences that have been observed after melatonin administration, and they may be due to its effect on mitochondrial physiology [44–46], namely, antioxidant and free-radical scavenging properties that preserve the stability, integrity, and function of the mitochondrial membrane. ROS-induced alterations to mitochondrial membrane may be a contributory factor in a variety of pathological conditions including heart ischemia-reperfusion, aging, and age-related cardiovascular and neurodegenerative diseases [47]. Experimental studies have shown that serum levels of melatonin significantly decrease in aged animals compared with young animals [48, 49], and it has been suggested that, in humans, melatonin contributes to the total antioxidant capability of serum [50]. It has been hypothesized [51] that the increase of oxidants may lead to alteration of physical and cognitive functions that are typical features of the aging process. Oxidants accumulate in the body, leading to an increase in oxidative stress that, in turn, can also imply the induction of protective and survival functions.

Centenarians, a widely used model of successful aging, are less prone to oxidative stress and have been found to be in possession of better antioxidant defenses than younger elderly cohorts [52]. These findings have also been confirmed in another report stating that this particular category of individuals show differences in antioxidant defenses (mainly plasma vitamin E) and fasting plasma glucose that seem to provide a contribution to the genesis of oxidative stress and to the differences between other elderly people and centenarians. The same authors report that it is possible that the difference in antioxidant defense between aged subjects and centenarians is due to a particular diet composition, since centenarians have demonstrated an elevated daily protein intake and a diet consisting mainly of vegetables, which are the main natural source of antioxidants, for example, vitamins C and E [53]. A higher antioxidant activity has also been observed in a study comparing chronic diseases and other related health indicators of centenarians with other age groups in longevity areas in China [54]. Okinawan people are famous for their long average life expectancy, high numbers of centenarians, and low risk of age-associated diseases. Their diet is heavy with fruit, that is, rich in phytonutrients and antioxidants, but reduced in meat, refined grains, saturated fat, sugar, salt, and full-fat dairy products. The traditional Mediterranean diet and the modern DASH

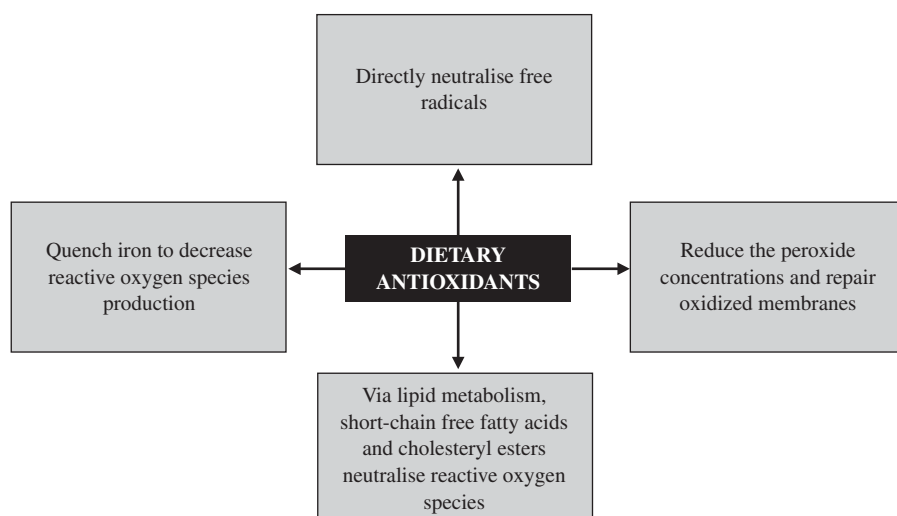


Fig. 15.2 Dietary antioxidants' main functions.

(Dietary Approaches to Stop Hypertension) diet share many characteristics with the Okinawan diet. Features such as the low levels of saturated fat, high antioxidant intake, and low glycemic load in these diets are likely to contribute to a decreased risk of cardiovascular disease, some cancers, and other chronic diseases through multiple mechanisms, including reduced oxidative stress. A comparison of the nutrient profiles of the three dietary patterns shows that the traditional Okinawan diet is the lowest in fat intake, particularly in terms of saturated fat, and the highest in carbohydrate intake, in keeping with the very high intake of antioxidant-rich yet calorie-poor orange-yellow root vegetables, such as sweet potatoes, and green leafy vegetables. The high longevity that characterizes this population is thought to be related to a healthy lifestyle, particularly to the traditional diet that is low in calories but rich in nutrients, especially with regard to phytonutrients in the form of antioxidants and flavonoids [55]. The main function of dietary antioxidants are summarized in Figure 15.2, while the main antioxidants, their mechanisms of action, and their main dietary sources are summarized in Table 15.1.

15.3 CARDIOVASCULAR DISEASE, RISK, AND ISCHEMIA-REPERFUSION INJURY

Coronary heart disease and stroke have been associated to several risk factors, namely, tobacco use, alcohol use (having 1–2 alcohol drinks a day may lead to a 30% reduction in heart disease, but above this level alcohol consumption will damage the heart muscle), high blood pressure (hypertension), obesity and related comorbidities (metabolic syndrome, diabetes, and so on), physical inactivity (increases the risk of heart disease and stroke

by 50%), and unhealthy diet (excessive consumption of saturated fat increases the risk of heart disease and stroke; it is estimated to cause about 31% of coronary heart disease and 11% of stroke worldwide). Generally it has been pointed out that abnormal blood lipid levels (hyperlipemia), in the form of high total cholesterol, high levels of triglycerides, and high levels of low-density lipoprotein or low levels of high-density lipoprotein (HDL) cholesterol, increase the risk of heart disease and stroke. Moreover, the contraceptive pill and hormone replacement therapy (HRT) may increase the risk of heart disease [56]. Nowadays, oxidative species seem to play a key role in these pathological conditions. Relevant evidence has supported the theory that free radical-mediated oxidative processes and specific related products play a key role in atherogenesis [3].

Aging has also been related to an increased risk of stroke, in fact the latter doubles every decade after the age of 55. Family history of cardiovascular disease is also a risk indicator. A man is at greater risk of heart disease than a premenopausal woman. Ethnic origin plays a role, too. People with African or Asian ancestry are at higher risk of developing cardiovascular disease than other racial groups [56]. Several studies have linked a high consumption of plant polyphenols with a decreased risk of cardiovascular disease and hypertension [57–61]. Vitamin C (500 mg twice a day) and vitamin E (400 IU twice a day), administered for 1 year, reduce the progression of cardiac transplant-associated arteriosclerosis (TxAA) in patients with normal or abnormal endothelial function, with a particular benefit in patients with endothelial dysfunction [62]. Antioxidant vitamin intake [vitamins A, C, and E, 1683 mg (± 1245), 371 mg (± 375), and 97 mg (± 165) respectively] does not seem to be significantly related to coronary artery calcification, implying that

TABLE 15.1 Common antioxidants, their action, and main dietary sources

Antioxidant	Action	Foods containing antioxidant
Vitamin A (beta-carotene)	Protection against lipid oxidation	Variety of dark orange, red, yellow, and green vegetables and fruits such as broccoli, kale, spinach, sweet potatoes, carrots, red and yellow peppers, apricots, cantaloupe, and mangos
Vitamin C (ascorbic acid)	Inhibition of reactive oxygen species. Stimulation of the vitamin E antioxidant power and selenium. Protection against damages caused by LDL-ox	Citrus fruits and their juices, berries, dark green vegetables (spinach, asparagus, green peppers, brussels sprouts, broccoli, watercress), red and yellow peppers, tomatoes and tomato juice, pineapple, cantaloupe, mangos, papaya, and guava
Vitamin E (α -tocopherol)	Protection of membrane polyunsaturated fatty acids LDL peroxidation	Vegetable oils such as olive, soybean, corn, cottonseed and safflower, nuts and nut butters, seeds, whole grains, wheat, wheat germ, brown rice, oatmeal, soybeans, sweet potatoes, legumes (beans, lentils, split peas), and dark leafy green vegetables
Cu, Zn, Mn, Se	Cofactors of antioxidant enzymes SOD-Cu/Zn, SOD-Mn and glutathione peroxidase	Brazil nuts, brewer's yeast, oatmeal, brown rice, chicken, eggs, dairy products, garlic, molasses, onions, salmon, seafood, tuna, wheat germ, whole grains, and most vegetable.
Other carotenoids (lycopene)	Protection against lipid oxidation, LDL, proteins, and DNA; elimination and inactivation of free radicals	Tomato, ketchup, hot pepper, fishes, watermelon, grapefruit
Resveratrol, catechin, quercetin, phenolic acid (phytochemicals)	Protection against lipid and DNA oxidation	Grapes, berries, peanuts, pine nuts

there is no effect on the development of early coronary atherosclerosis. Vitamin E, according to this study, is positively associated with an increased risk of calcified atherosclerosis [63]. Intravenous infusion of Edaravone, a powerful neuroprotective free radical scavenger, has been investigated in 141 patients with cardioembolic stroke, and it was concluded that it may be only effective in patients with mild cardioembolic stroke [64]. Supplementation with antioxidant vitamins and B-group vitamins, separately or together [96 acute ischemic stroke patients randomized to receive either daily oral 727 mg vitamin E and 500 mg vitamin C ($n=24$), B-group vitamins (5 mg folic acid, 5 mg vitamin B2, 50 mg vitamin B6, and 0.4 mg vitamin B12; $n=24$), both vitamins together ($n=24$), or no supplementation ($n=24$) for 14 days], enhance antioxidant capacity, mitigate oxidative damage, and may have an antiinflammatory effect immediately after an acute ischemic stroke [65]. Administration of 15 g *N*-acetylcysteine (NAC) infused over 24 h, in combination with streptokinase, significantly diminishes oxidative stress and improves left ventricular (LV) function in patients with acute myocardial infarction (MI) [66]. In addition, serum carotenoids have been

associated, some interactively with smoking, with some benefits such as markers of inflammation, oxidative stress, and endothelial dysfunction [67]. It has also been proposed that antioxidant supplementation (vitamins E and C) may be able to counteract the progressive oxidative stress associated with Chagas disease, underlining that future perspectives for treatment of Chagas disease might include an antioxidant therapy in order to attenuate the consequences of oxidative insult related to this disease [68]. Administration of vitamin C (1 g) and vitamin E (400 IU) for 8 weeks in untreated essential hypertensive patients improves flow-mediated dilation (FMD), significantly reduces central pulse wave velocity (PWV), and decreases augmentation index (Aix). This treatment also increases plasma vitamin levels and antioxidant capacity and levels of oxidative stress. The decrease and changes in central PWV have been related to changes in levels of oxidative stress. Therefore this treatment has beneficial effects on endothelium-dependent vasodilation and arterial stiffness in these particular patients [69]. In a study involving 19 patients with heart failure (HF) after MI, administration of vitamin C has proven to enhance the contractile response to

dobutamine and improve myocardial efficiency [70]. A double-blind parallel study, consisting of five weeks of 100 mg/day or 200 mg/day gamma-T supplementation, has shown that gamma-T supplementation seems to have a permissive role in decreasing the risk of thrombotic events by improving lipid profile and reducing platelet activity [71]. Interestingly, it has been reported that edaravone can salvage the boundary zone of the infarct and is a useful cytoprotective antiedema agent, as shown by magnetic resonance imaging in six patients with extensive hemispheric ischemic stroke [72]. Three months of lipoic acid supplementation seems to relieve exercise pain according to a randomized, double-blind controlled study in 28 participants with peripheral arterial disease (PAD) [73]. Vitamin E (400 U/day) supplementation appears to reduce cardiovascular events in individuals with diabetes mellitus (DM) and Haptoglobin (Hp) 2-2 genotype, a subgroup that comprises 2–3% of the general population [74]. A randomized placebo-controlled study in 26 healthy male subjects and eight male patients with PAD has shown that ischemia-reperfusion (IR)-induced vascular injury can be prevented by administration of vitamin C [75]. A randomized double-blind placebo-controlled clinical trial conducted in 110 men with grade 1 essential hypertension (EH) (35–60 years of age without obesity, dyslipidemia, and diabetes mellitus, nonsmokers, not undergoing vigorous physical exercise, without the use of any medication and/or high consumption of fruit and vegetables) who were randomly assigned to receive either vitamins C + E [vitamin C (1 g/day) plus vitamin E (400 IU/day)] or placebo for 8 weeks has shown that enhancement of antioxidant status by supplementation with vitamins C and E in patients with EH is associated with lower blood pressure [76]. The effects of AGI-1067 on coronary atherosclerosis has been investigated in a placebo-controlled, randomized trial. Atherosclerosis regression was observed, although it was not significantly different from placebo. The antiinflammatory effect of AGI-1067 was supported by reduced levels of myeloperoxidase [77]. An extensive review [78] has shown that dietary advice reduces total serum cholesterol and LDL cholesterol after 3–24 months. It also reduces blood pressure and 24-hour urinary sodium excretion after 3–36 months. Furthermore, dietary advice increases fruit and vegetable intake by 1.25 servings/day. Dietary fiber intake increases with advice by 5.99 g/day, while total dietary fat, as a percentage of total energy intake, falls by 4.49% with dietary advice. In addition, saturated fat intake falls by 2.36%. This study points out that dietary advice may be an effective tool to achieve modest beneficial changes in diet and cardiovascular risk factors over approximately 10 months. Melatonin has significant protective action against the cardiac damage and altered physiology that occur during IR injury

[79–81]. Furthermore, it has been demonstrated that melatonin, at pharmacological concentrations, strongly protects against IR myocardial damage [82]. This protective effect of melatonin may be explained through its action at the mitochondrial level.

15.4 ROS AND NEURODEGENERATIVE DISORDERS

Oxidative stress is involved in the pathogenesis of neurodegenerative diseases and, in particular, the central nervous system (CNS) can be targeted by free radicals because of its high metal content, leading to the formation of ROS and to the relatively low content of antioxidant defenses. The brain seems to be particularly targeted by ROS since it consumes approximately 20% of total body oxygen. It contains high levels of PUFA and may contain low proportions of endogenous antioxidants. Moreover, iron accumulates in brain-specific regions, and iron-binding proteins, such as ferritin, may be relatively deficient in them [83]. Specifically, PUFA are easily targeted by lipid peroxidation because of the ROS interaction with them. This can be explained by the fact that the increase in the number of the molecule double bonds is related to the ease of the hydrogen atom removal. It is not clear yet whether oxidative damage causes neurodegeneration or what its consequences are.

Unregulated metal metabolism may lead to oxidative stress that, in turn, leads to neuronal cell death, but metals play a key role in cellular metabolism and cell signaling. Mutations in mtDNA or metal overload in aged brain lead to oxidative stress and free radical-mediated pathological changes in neurons. Neuronal proteins and structural components are modified because of oxidative stress in different neurological disorders, leading to neuroinflammation and loss of cognitive function in Alzheimer disease (AD), Parkinson disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) [84]. Increased oxidative stress has been linked to AD, MS, Batten disease, PD, and brain tumors [85]. In particular, AD is characterized by memory loss with age, a symptom that is linked to ROS-induced neuron damage. AD has been first reported by Alois Alzheimer, a German psychiatrist and neuropathologist, a hundred years ago, and it is the most common course of dementia in the elderly above 65 years of age. It is characterized by two distinct pathological features, namely, intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques in the brain, which are both related to neuronal demise and consequential onset of dementia symptoms. NFTs contain abnormally hyperphosphorylated forms of the microtubule-binding

protein tau. Amyloid plaques are made up of insoluble aggregates of amyloid β ($A\beta$) peptides [86]. Clinical characteristics are memory dysfunction, loss of lexical access, spatial and temporal disorientation, and impairment of judgment. The neurotransmitter acetylcholine, necessary for cognition and memory, and oxidative stress may play an important role in AD development [87]. The complex nature and genesis of oxidative damage in AD can be partly due to mitochondrial and redox-active metal anomalies. AD is essentially an acceleration of the aging mechanism in affected brain regions that progressively become more damaged by free radicals. Neurodegeneration, occurring in specific brain areas, namely, substantia nigra and striatum, plus dopamine depletion is considered the key feature. In addition, increased oxidative stress, abnormal mitochondrial function, and excitotoxicity are among the most relevant initiators or mediators of neuronal damage. The disease is inherited and familiar (FALS) in 10% of all ALS cases. About 20% of FALS cases are associated with mutations and lowered activity of CuZnSOD, which is known to catalyze the formation

of hydrogen peroxide through the dismutation of superoxide radical anions, playing a relevant role in regulating oxidative damage to cells. Oxidative damage to DNA, protein, and lipids has been observed [83]. The oxidation of arachidonic and docosahexanoic acids, which are the main constituents of the brain membrane phospholipids, leads to the production of malondialdehyde (MDA) and 4-hydroxynonenal, which increase in autopsied specimens from multiple brain regions and in CSF in AD subjects [88–90]. Advanced glycation end products have been found present in AD brains and closely associated with the senile plaques [91].

Vitamin E is a lipid-soluble, chain-breaking, natural antioxidant that is able to cross the blood-brain barrier and accumulate in the CNS, where it reduces markers of oxidative stress [92; see Fig. 15.3 for other properties and its interaction with vitamin C]. A study performed in more than 4000 elderly patients has shown that decreased circulating levels of vitamin E are consistently associated with decreasing memory levels, while the same association has not been observed for plasma levels of vitamins

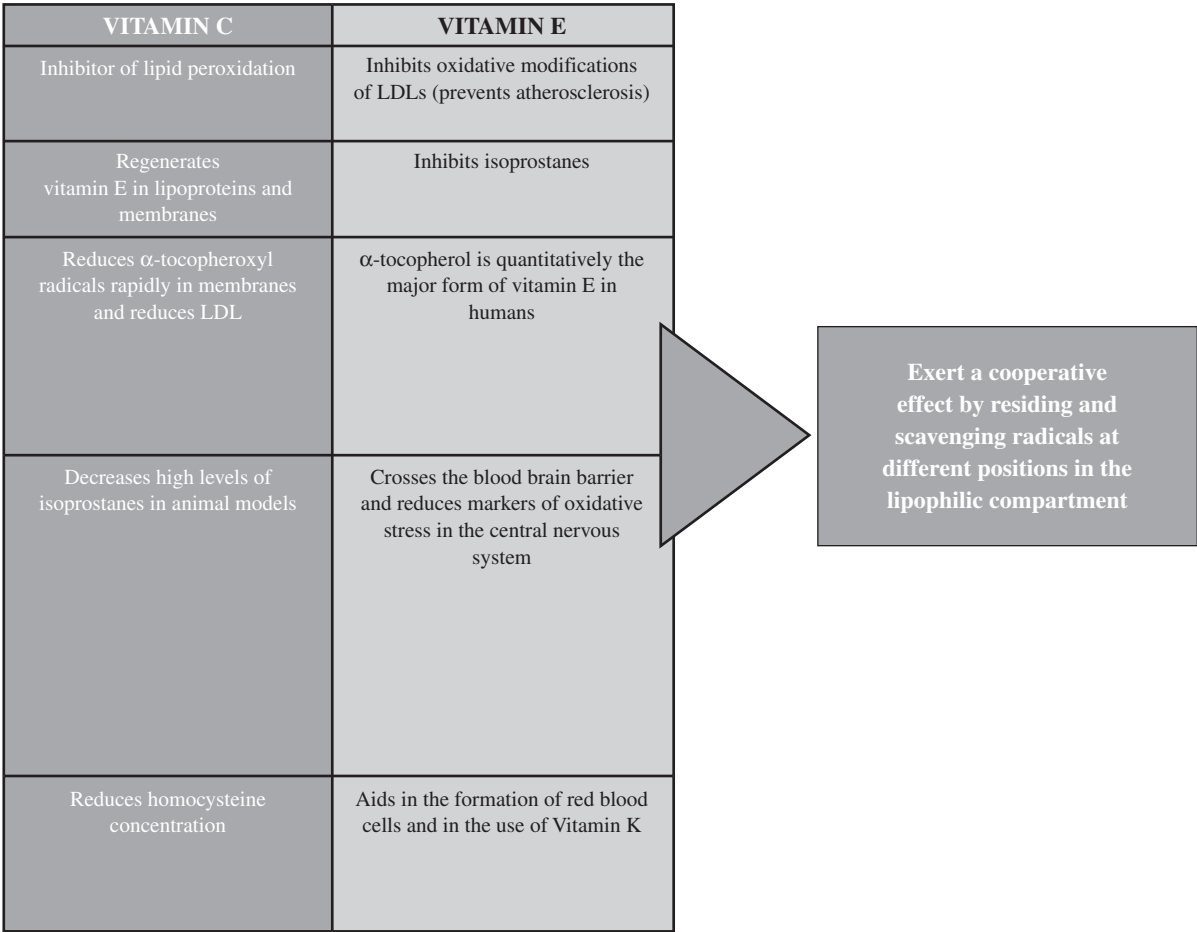


Fig. 15.3 Vitamin C and vitamin E main characteristics and functions [227–229].

A and C and β -carotene [93]. The Honolulu–Asia Aging Study has shown that supplementary intake of vitamins E and C are both associated with better cognitive performance [94]. The Chicago Health and Aging Project, which has analyzed almost 3000 subjects aged 65 to 102 years, has shown that supplementary or dietary intake of vitamin E, but not C, is inversely correlated with cognitive decline [95]. In addition, the Nurses' Health Study, which includes almost 15,000 women aged 70–79 years, has shown that long-term use of vitamin C and E supplements results in a better cognitive status [96]. A lower risk of developing AD [97] has been associated with dietary intake of vitamin E. Another study found that dietary and not supplement-derived vitamin E intake is associated with a lower risk of AD only in individuals who were not carriers of apolipoprotein E4 [98]. Another report showed no association between dietary or supplementary vitamin E intake and a decrease in the risk for AD [99]. The Cache County study also reported no benefit or significant reduction in the risk of AD from supplementation of vitamin E alone. However, the beneficial effect of a reduced risk of developing AD is seen in the combination of vitamin E and vitamin C [100]. A subgroup of the Honolulu–Asia Aging Study, which examined dietary intake of antioxidants and risk of late-life dementia, showed that vitamin C, vitamin E, β -carotene, and flavonoids are not associated with a reduced risk of dementia [101]. In 1997 the results of a double-blind, placebo-controlled 2-year randomized multicenter trial in 341 patients with moderate AD who received the selective monoamine oxidase inhibitor selegiline (10 mg/day), α -tocopherol (vitamin E, 2000 IU/day), both selegiline and α -tocopherol, or placebo were reported. This study shows that treatment with selegiline or α -tocopherol slows the progression of disease [102]. Recently, in a double-blind 3-year-follow-up study, the same high doses of vitamin E had no benefit in subjects with a clinical diagnosis of mild cognitive impairment (MCI). Consequently, the subjects receiving the antioxidants did not show any difference from placebo in preventing the progression of MCI to AD [103]. The same authors have also shown that in a subgroup of these patients the MRI-annualized percent changes of the volumes for some areas of the brain (hippocampus, entorhinal cortex) are less evident in the group receiving the vitamin E than in the placebo group [104].

Among other antioxidants, selenium (Se) seems to be involved in the maintenance of brain function and is particularly present in the brain, especially in the gray matter (area responsible for chemical synaptic communication), even with prolonged Se dietary deficiency [105]. Cognitive impairment, depression, anxiety, and hostility have been associated with low body levels of Se in humans [106]. Levels of Se are elevated in amygdala

[107] and microsomes in the temporal lobe of patients with AD [108]. Selenoproteins, such as GPX1, GPX4, SelP, thioredoxin reductases, selenoprotein W (SelW), and selenoprotein M (SelM), have been implicated in brain maintenance and may play a protective role in several neurodegenerative conditions [109]. Patients affected by AD have shown a significant age-dependent decrease in the Se-dependent glutathione peroxidase activity in plasma and red blood cells [110], while another study has shown an increase in thioredoxin reductase activity in patients affected by the same disease [111]. Interestingly, another study has shown that the levels of Se in plasma and CSF from patients affected by AD are comparable to normal individuals [112]. In the future it would be interesting to investigate the role of selenoproteins in neurological aging and their potential interaction with telomeres. In fact, the length of the telomeres in cells, derived from the hippocampus, has been proven to be longer in AD patients than in control individuals, while in buccal and white blood cells from the same AD patients the telomere lengths are significantly shorter than those of the control groups [113]. This shows that AD patients have evolved a unique mechanism for telomere maintenance. Tanaka and colleagues [114] have shown that age-dependent telomere shortening in human brain microvascular endotheliocytes is negated by phosphorylated α -tocopherol, possibly through α -tocopherol's ability to decrease intracellular oxidative stress. Se, in the form of selenite, prevents telomere length shortening during cellular aging in the normal human L-02 hepatocyte [115].

PD is a chronic, progressive neurodegenerative disorder that affects at least 1% of people by the age of 70. This disease was first described by James Parkinson in his 1817 monograph "An Essay on the Shaking Palsy." After that, Charcot described the cardinal clinical features of PD, namely, rest tremor, rigidity, balance impairment, and slowness of movement [116]. The majority of the movement-related symptoms of PD are caused by a lack of dopamine due to the loss of dopamine-producing cells in the substantia nigra. As a consequence, communication between the substantia nigra and corpus striatum becomes impaired, and so does movement. Among others, flavonoids possess potential neuroprotective mechanisms. Their antiinflammatory properties added to their ability to act as scavengers for ROS to maintain the correct glutathione levels and to inhibit Ca^{2+} influx, which represents the end of the cell death cascade, make them important candidates for treatment of AD and PD [117, 118].

We have previously described melatonin's antioxidant properties. PD, together with other age-related neurodegenerative disorders, have been associated with a malfunction of mitochondrial complex I [119, 120],

strongly suggesting that melatonin, through the prevention of complex I deficiency, may improve mitochondrial physiology in brain aging and age-associated brain diseases. The effects of melatonin in the field of cognitive impairment or dementia have been summarized in a review that collected all randomized controlled trials involving the use of orally administered melatonin in any dosage compared with a control group. Unfortunately, the conclusion was that there is insufficient evidence to support the effectiveness of melatonin in managing the cognitive and noncognitive sequelae of dementia [121]. Experimental studies have suggested that mitochondrial decay is a major contributor to brain tissue alterations associated with aging [122], and a potential role of melatonin in mitigation of changes associated with brain aging has been observed [123, 124], but conclusive clinical data are not available yet. A randomized controlled trial involving 16 patients receiving 1 hour of morning light exposure Monday to Friday for 10 weeks and 5 mg melatonin (LM) compared to a placebo group receiving usual indoor light has been performed. The following results were reported: significant improvement in daytime somnolence consisting in a reduction in daytime sleep duration, an increase in daytime activity, an improvement in day-night sleep ratio, and a significant increase in rest-activity rhythm amplitude. Unfortunately, this study does not confirm that the improvements can be attributed to melatonin [125]. A large study has shown that the use of vitamins E and C, alone or in combination, did not reduce the risk of AD or overall dementia over 5.5 years of follow-up, and therefore their use is not recommended for prevention of dementia in older adults [126]. Tocopherol, CoQ₁₀, and glutathione have been used in the prevention or treatment of PD, and their effectiveness has been related to their potential to alter the course of two common theories of PD pathogenesis, namely, free radical generation and mitochondrial complex I deficiency. A collection of three large clinical studies involving the use of tocopherol, four trials of CoQ₁₀, and a study of glutathione for the prevention and treatment of PD has shown that antioxidant supplementation, in particular tocopherol, does not alter the course of PD. CoQ₁₀ and glutathione, according to the studies analyzed, have shown a statistically significant improvement in PD symptoms [127]. A randomized, double blind, placebo-controlled study evaluated the effect of melatonin on sleep and motor dysfunction in PD. This study involved 18 patients with motor dysfunction randomized to receive melatonin (3 mg) or placebo 1 hour before bedtime, for 4 weeks. Melatonin significantly improved the subjective quality of sleep, but polysomnography (PSG) abnormalities were not changed. Motor dysfunction was not improved by the use of melatonin [128].

15.5 COMPLEX REGIONAL PAIN SYNDROME

Complex regional pain syndrome (CRPS) was described for the first time more than a hundred years ago, and it is characterized by chronicity and relapses that can result in significant disability over time. Fractures and surgical insult are considered the main triggers for CRPS, but it can also develop after a seemingly benign trauma. We still do not fully understand how this condition works. It goes by many names, “reflex Sympathetic Dystrophy,” “causalgia,” “Sudeck atrophy,” “algodystrophy,” “neurodystrophy,” and “posttraumatic dystrophy.” The term CRPS was adopted in 1995 by the International Association for the Study of Pain (IASP) to standardize the taxonomy [129–131]. On the basis of signs and symptoms of CRPS, four subgroups have been created: (1) a unique set of signs and symptoms indicating abnormalities in pain processing, for example, allodynia and hyperalgesia; (2) a different set of clinical signs characterized by skin color and temperature changes, which are indicative of vasomotor dysfunction; (3) a third group characterized by edema and sudomotor dysfunction, for example, sweating changes; and (4) a fourth subgroup including motor and trophic signs and symptoms [132]. The progression of CRPS involves three stages: (1) acute, warm, or inflammatory; (2) dystrophic; and (3) atrophic. MRI findings of diffuse bone marrow edema on both sides of the joint, accompanied by periarticular soft tissue edema, with or without a joint effusion, are present in about half of all patients in CRPS stage 1 [133]. It has been hypothesized that ROS may contribute to CRPS. Experimental studies have shown that *N*-acetyl-L-cysteine and 4-hydroxy-2,2,6,6-tetramethylpiperidine reduce the signs of hyperalgesia and allodynia in animal models of CRPS [134]. In addition, topical treatment with 50% dimethyl sulfoxide cream can be effective in decreasing the hypoxia-related production of free oxygen radicals [135]. The use of vitamin C in CRPS has been widely investigated. A double-blind, prospective multicenter trial involving 416 patients with 427 wrist fractures randomly allocated to placebo or treatment with 200, 500, or 1500 mg of vitamin C daily for 50 days showed that vitamin C reduces the prevalence of CRPS after wrist fractures, and a daily dose of 500 mg for 50 days is recommended [136]. It has been reported that a 5-year-old girl with clinical and radiographic evidence of scurvy who developed features of CRPS 2 years after a left ankle fracture improved with the administration of vitamin C [137].

Zollinger and colleagues [138] performed 32 arthroplasties for first carpometacarpal arthritis in 27 patients, using a cementless total trapeziometacarpal joint prosthesis that may be complicated by CRPS. All their patients took vitamin C 500 mg/day starting 2 days

before surgery and continuing for 50 days. There were no cases of CRPS under vitamin C prophylaxis. Besse et al. [139] studied a group of patients having surgery on the foot or ankle with the exception of diabetic foot cases. Four hundred and twenty feet were included in this study: 185 in Group I (no Vitamin C treatment), and 235 in Group II (1 g/day of preventative oral vitamin C treatment). CRPS I occurred in 18 cases in Group I (9.6%) and 4 cases in Group II (1.7%) (57). The authors conclude that vitamin C is effective in preventing CRPS I of the foot and ankle since it was not an infrequent complication in their control group (9.6%). It has been hypothesized that both inflammatory and neural mechanisms may contribute to CRPS type I (CRPS-I). The levels of antioxidants in the serum and saliva of 31 patients with CRPS-I and in a control group of 21 healthy volunteers have been investigated, showing that serum lipid peroxidation products (MDA) and all antioxidative parameters analyzed were significantly elevated in CRPS-I patients. Median salivary peroxidase and superoxide dismutase (SOD) activity values, uric acid (UA) concentration, and total antioxidant status (TAS) values were higher in CRPS I patients by 150%, 280%, 60%, and 200%, respectively, as compared with control subjects. Oxidative changes were also found in the serum, namely, mean serum UA and MDA concentrations, and TAS value in the CRPS-I patients were higher by 16%, 25%, and 22%, respectively, than in the control group. Median salivary albumin concentration and median salivary LDH activities in the patients were 2.5 times and 3.1 times higher than in the control group. The accumulated data show that free radicals are involved in the pathophysiology of CRPS I, which is reflected both in serum and salivary analyses [140].

15.6 CANCER

The Hippocratic “corpus” consists of 70 books written by Hippocrates of Cos (460 BCE–370 BCE) and other physicians. It was the first text to use the words *karkinos* and *karkinoma* to describe a nonhealing swelling or ulceration and a malignant nonhealing tumor, respectively. Hippocrates also introduced the word *scirrhus* to describe hard tumors [141]. The word “tumor” derives from the Latin word *tumere* meaning “to swell.” It is synonymous with the term “neoplasm” and can be defined as a lesion resulting from the autonomous or relatively autonomous abnormal growth of cells that persists after the initiating stimulus has been removed [142]. ROS formed in vivo are powerful oxidizing agents, capable of damaging DNA and other biomolecules. An increased formation of the molecules can promote the development of malignancy [143]. Histone deacetylase

inhibitors are important regulators of many oxidative stress pathways, including those involved in the cellular response to oxidative stress. Aberrant regulation of these pathways by histone deacetylases may play a critical role in cancer progression [144]. The relationship among lipid soluble antioxidant vitamins, lipid peroxidation, disease stage, and systemic inflammatory response has been studied in 14 healthy subjects, 20 patients with benign prostate hyperplasia (BPH), and patients with localized ($n = 40$) and metastatic ($n = 38$) prostate cancer. This study suggests that in patients with prostate cancer lower concentrations of carotenoids, in particular, lycopene, reflect a disease progression rather than a systemic inflammatory response [145].

An interesting study evaluated the association between the intake of antioxidants from foods and supplements and the risk of prostate cancer among men in the screening arm of the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. This study does not provide strong support for population-wide increase of high-dose antioxidant supplementation for the prevention of prostate cancer. However, vitamin E supplementation in male smokers and beta-carotene supplementation in men with low dietary beta-carotene intake are associated with a reduced risk of this disease [146]. The relationship between Se supplementation and prevalent and incident colorectal adenomas and colorectal cancer (CRC), detected during the Nutritional Prevention of Cancer trial follow-up, has been studied in 1312 recipients randomized to 200 mcg of selenized yeast [only 598 underwent endoscopic screening (flexible sigmoidoscopy or colonoscopy) for CRC sometime during the follow-up period]. This study suggests that Se supplementation is associated with a significantly reduced risk of prevalent adenomas, but only among subjects with a low baseline Se level or among current smokers [147]. A study performed in French adults (7876 women and 5141 men) who were randomized to take an oral daily capsule of antioxidants (120 mg vitamin C, 30 mg vitamin E, 6 mg beta-carotene, 100 µg Se, and 20 mg zinc) or a matching placebo has shown that antioxidant supplementation affects the incidence of skin cancer (SC) differentially in men and women. In fact, in women the incidence of SC was higher in the antioxidant group, while in men the incidence did not differ between the two treatment groups [148]. A study in 1732 Finnish male smokers who were diagnosed with incident prostate cancer has evidenced that higher pre-diagnostic serum concentrations of α -tocopherol, but not dietary vitamin E, are associated with a lower risk of developing prostate cancer, particularly advanced prostate cancer [149]. A systematic review, searching multiple electronic databases from their dates of inception until August 2005, has evidenced that beta-carotene

supplementation increases cancer incidence and cancer mortality among smokers, whereas vitamin E supplementation has no effect. Moreover, Se supplementation might have anticarcinogenic effects in men, and therefore it requires further research [150]. A review including all the randomized-controlled trials of Se monosupplements in cancer patients undergoing tumor specific therapy, namely, chemotherapy, radiotherapy, or surgery, identified only two trials. The first of these investigated secondary lymphedema, while the second one investigated radiotherapy-induced diarrhea as a secondary outcome. The trial on secondary lymphedema reported a decreased number of recurrent erysipela infections in the Se supplementation group compared to placebo. The ongoing trial on radiotherapy-associated diarrhea preliminarily reported a lower incidence of diarrhea in patients receiving Se supplementation concomitant to pelvic radiation. According to this review, at present there is insufficient evidence that Se supplementation alleviates the side effects of tumor-specific chemotherapy or radiotherapy treatments [151].

15.7 ASSOCIATION BETWEEN ROS AND VARIOUS DISEASES

Oxidative stress is one of the potential biochemical mechanisms involved in the pathogenesis of NASH [152], and it seems to play a key role in alcoholic steatohepatitis [153]. Thyroid specimens from patients with Graves disease, follicular adenoma, and papillary and follicular carcinomas contain significantly higher concentrations of xanthine oxidase (XOD) and GSH-PX, compared to those in normal thyroid tissue [3]. Redox status imbalance and a change in the lipid profile are the final result of a lack of estrogens that characterizes the menopause. Moreover, the symptoms accompanying the menopause, for example, hot flushes, suggest an increased metabolic activity [154] that may lead to redox status imbalance toward oxidative processes. Oxidative damage, because of the testicular venous backflow, may represent one of the causes of gonad injury and seems to precede the histological alteration typical of varicocele and consequent male infertility [155, 156]. The lung is continuously exposed to a relatively high oxygen tension, pollutants, and metabolic products derived from them. The inhalation of cigarette smoke, ozone, carcinogens and other chemicals, and dust particles is able to further increase ROS and RNS in the lung and may lead, in time, to depletion of endogenous antioxidants [157]. Oxidative stress also plays a relevant pathogenetic role in human immunodeficiency virus (HIV) infections. In fact, in the early phase of the disease, it has been observed that serum and tissue

antioxidants levels are low, while peroxidation products are elevated. In addition, high plasma levels of MDA, reduced plasma GSH, decreased GSHPx (glutathione peroxidase) and SOD activities are also found. HIV infection also results in considerably reduced vitamin E and C concentrations and very low plasma Zn and Se levels. In particular, Se deficiency is related to the occurrence, virulence, and disease progression of some virus infections, including HIV progression to AIDS [158].

Several factors are involved in the development of oxidative stress in the joints of rheumatoid arthritis patients. ROS generation from locally activated leukocytes is followed by a pressure increase in the synovial cavity [159], a capillary density reduction, vascular changes, and an increased metabolic rate of synovial tissue. A randomized prospective study was conducted to compare patients receiving α -tocopherol and ascorbate to those receiving standard care. Five hundred ninety-five patients were enrolled and analyzed (91% were victims of trauma). It was concluded that the early administration of α -tocopherol and ascorbic acid reduces the incidence of organ failure and shortens ICU length of stay in this cohort of critically ill surgical patients [160]. Endometriosis is associated with a general inflammatory response in the peritoneal cavity, and oxidative stress may represent a potential factor involved in its pathophysiology. Endothelial nitric oxide synthase, the enzyme that produces NO, is also overexpressed in endometriosis and adenomyosis. The endometrium shows altered expression of enzymes such as SOD and GSHPx involved in defense against oxidative stress. Also, vitamin E levels are also significantly lower in the peritoneal fluid of women with endometriosis in the presence of redox-active metal ions, as estrogens are established oxidants. This mechanism might be due to the estrogen proinflammatory effect; in fact, the results of hormone therapy are correlated with an increased amount of CRP, a known marker of inflammation [161]. Therefore, estrogens and their metabolites have both prooxidant and antioxidant properties depending on the availability of metal ions and/or their dose and formulation. *Helicobacter pylori* is an important agent in the pathogenesis of active chronic gastritis, peptic ulcer, and low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma and in gastric carcinogenesis [162]. Generation of cytotoxins, urease, ammonia, T cell-mediated damage and a mainly humoral reaction are among the events that involve mucosal integrity following *H. pylori* infection [163]. Moreover, reactive oxygen metabolites (ROMs) have been found to play a relevant role in gastroduodenal inflammatory damage [164]. In particular, the acute mucosal inflammatory infiltrate (e.g., polymorphonuclear cells, PMNs) that characterizes *H. pylori*-related chronic active gastritis could

be an important source of free radicals [165], as both in vivo and in vitro studies have reported a positive relation between *H. pylori* infection and ROM generation [164, 166]. A review [167] including six randomized clinical trials has examined the effects of cysteine, cystine, or *N*-acetylcysteine supplementation of neonatal growth under parenteral nutrition (PN), showing that:

- Nitrogen retention is significantly increased by cysteine supplementation.
- Plasma levels of cysteine are significantly increased by cysteine supplementation but not by *N*-acetylcysteine supplementation.
- There is insufficient evidence to assess the risks of cysteine supplementation, especially regarding metabolic acidosis, which has been reported during the first 2 weeks of cysteine chloride administration.

An analysis of 23 studies based on the use of antioxidant supplements in amyotrophic lateral sclerosis (SLA) has shown no significant effect for vitamin E 500 mg/twice a day, vitamin E 1 g/five times a day, acetylcysteine 50 mg/kg daily subcutaneous infusion, or a combination of L-methionine 2 g, vitamin E 400 IU, and Se 0.03 mg administered three times a day [168].

PUFA seem to have no major effect on the main clinical outcome in MS (disease progression) and do not substantially affect the risk of clinical relapses over 2 years [169].

15.8 PREGNANCY AND PREECLAMPSIA

High blood pressure and proteinuria occurring after 20 weeks of pregnancy and affecting both the mother and the unborn baby are the main features of preeclampsia. From a clinical perspective this pathological condition is characterized by swelling, sudden weight gain, headaches, changes in vision, generalized vasoconstriction, increased vasoactivity, reduced perfusion to organs, and platelet activation. Changes of the maternal vascular endothelium may play an important role in the development of the condition, and they have been correlated to a dysfunction in the antioxidant defenses. Reduced total omega-3 fatty acids, increased omega-6-to-omega-3 ratio, higher oxidative stress, and lower antioxidant levels have been observed in preeclamptic women. Similar characteristics have also been observed in cord samples. Therefore, a role for oxidative stress, leading to impaired essential PUFA levels, has been hypothesized in this condition, and antioxidant supplementation, along with PUFA, particularly omega-3 fatty acids, may be helpful in the management of preeclampsia [170]. According to a review dated 2008, no evidence

for an antioxidant routinely used in the treatment of preeclampsia has been observed [171]. Supplementation with 1000 mg vitamin C and 400 IU vitamin E (α -tocopherol) daily from the second trimester of pregnancy until delivery does not prevent preeclampsia in women at risk [172]. These findings have been confirmed by another randomized, controlled double-blind clinical trial showing that an antioxidant supplementation consisting of both vitamin C (1000 mg) and vitamin E (400 IU) does not reduce the rate of preeclampsia among patients with chronic hypertension or prior preeclampsia [173].

15.9 ASTHMA

Asthma is a chronic relapsing inflammatory disease of the airways. In adult patients, oxidative and nitrosative stress accompany acute asthma [174]. In an attempt to investigate the effects of antioxidant supplementation, it has been observed that patients who consumed more than 46.3 g/day of citrus fruit had a reduced risk of diagnosed and symptomatic asthma. In addition, the same study pointed out that dietary vitamin C and manganese were inversely and independently associated with symptomatic asthma, but only manganese was independently associated with diagnosed asthma. Moreover, plasma vitamin C levels were significantly lower in symptomatic cases than in control subjects [175]. The possible influx of diet modification in asthma has been addressed in a study involving 32 asthmatic adults who underwent a low-antioxidant diet for 10 days and then started a randomized crossover trial involving 3 \times 7-day treatment arms [placebo, tomato extract (45 mg lycopene/day), and tomato juice (45 mg lycopene/day)]. In asthmatic patients following a low-antioxidant diet, it was observed that plasma carotenoid concentrations decreased, Asthma Control Score worsened, %FEV and %FVC decreased, and %sputum neutrophils increased. After the 10 days of low-antioxidant diet, the patients who were treated with both tomato juice and extract showed a reduction in airway neutrophil influx, and treatment with tomato extract reduced sputum neutrophil elastase activity [176].

15.10 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a chronic disease characterized by incompletely reversible airflow limitation [177]. The oxidative inactivation of antiproteases, epithelial injury, increase in number of neutrophils in the pulmonary microvasculature, and gene expression of proinflammatory mediators seem to

play a key role in this condition. The antioxidant depletion or deficiency in antioxidants may contribute to oxidative stress. Furthermore, airflow limitation seems to be related to dietary deficiency of antioxidants. The effects of antioxidant polyphenol-rich pomegranate juice (PJ) supplementation for 5 weeks on patients with stable COPD has been investigated, but no benefit has been reported [178]. The association between antioxidant nutrients and markers of oxidative stress with pulmonary function in patients with chronic airflow limitation has been also investigated, showing that serum beta-cryptoxanthin, lutein/zeaxanthin, and retinol and dietary beta-carotene, beta-cryptoxanthin, lutein/zeaxanthin, vitamin C, and lycopene were positively associated with FEV1% (0.05, all associations). In addition, serum vitamins beta-cryptoxanthin, lutein/zeaxanthin, lycopene, dietary beta-cryptoxanthin, beta-carotene, and vitamin C were positively associated with FVC%. Erythrocytic glutathione was negatively associated with FEV1%, while plasma thiobarbituric acid-reactive substances (TBARS) were negatively associated with FVC% [179]. It has been observed that erdosteine treatment in current smokers with mild COPD leads to a significant drop in blood ROS and IL-8 in bronchial secretions (observed after day 4 from the beginning of treatment), while 8-isoprostane drop was significant only after day 10. An e-NO decrease was also reported, but it was not significant [180].

15.11 DIABETES TYPE 1 AND TYPE 2

Diabetes is a blood glucose metabolism impairment subdivided into type 1, which is due to the inability of the body to produce insulin, and type 2, when the body produces the insulin needed but the cells are not able to respond to it, making it ineffectual. Type 2 diabetes has been associated with obesity, and it is a condition that can lead to severe illness and even death. In the peripheral nervous system, diabetes causes a progressive deterioration of primarily sensory nerves, and the damage also extends to motor nerves. Approximately 50% of diabetics experience some degree of neuropathy, which is ultimately the leading cause of lower-extremity amputation [181]. In addition, variations in blood sugar level, namely, hypoglycemia or hyperglycemia can lead to atherosclerosis, neuropathy, retinopathy, and nephropathy. The hyperglycemic state that characterizes the diabetic patient is responsible for the high oxidative stress that accompanies this condition. In particular, this seems to be due to superoxide anion production by the cell glucose metabolism, which, in turn, is able to damage oxidative blood balance, leading to protein glycation and O_2 and H_2O_2 release. It has been shown

that high α -tocopherol levels, among patients with renal disease and in those using vitamin supplements are associated with lower coronary artery disease (CAD) risk in type 1 diabetes [182]. A randomized and open study [183] has been performed on a total of 36 patients with type 2 diabetes and hypercholesterolemia. They were randomly assigned to a probucol group (500 mg/day, $n=18$) or an atorvastatin group (10 mg/day, $n=18$). Over 3 months, total and LDL-cholesterol decreased significantly in both groups. LDL-cholesterol was significantly lower in the atorvastatin group than in the probucol group. HDL-C decreased significantly in the probucol group and did not change in the atorvastatin group. Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) decreased significantly in both groups after 3 months. Urinary 8-OHdG was significantly lower in the probucol group than in the atorvastatin group after the second and third months of administration.

A study involving 12 patients who were treated with oral ALA, 600 mg twice daily, over a period of 4 weeks has shown that short-term oral ALA treatment increases peripheral insulin sensitivity in patients with type 2 diabetes mellitus [184]. Cilostazol (100 mg) was administered to hypertensive type 2 diabetic patients twice daily. At 1 month follow-up the Cilostazol group (26 patients) showed significant decrease in hsCRP, erythrocyte sedimentation rate (38.7%), total leukocyte count, plasma MDA, HbA1c, and an increase in serum albumin and blood reduced glutathione from baseline. The cardiovascular risk assessment in terms of 10-year risk of cardiovascular heart disease (%), calculated using the United Kingdom Prospective Diabetes Study (UKPDS) guidelines, decreased by 6%. Therefore inflammatory and oxidative stress is high in hypertensive type 2 diabetic patients, and Cilostazol reduces those factors as well as coronary heart disease risk in diabetes mellitus [185].

A study involving 36 type 1 diabetic patients showed that neither normalization of glycemia nor vitamin C treatment alone was able to normalize endothelial dysfunction or oxidative stress; when insulin and vitamin C were combined, endothelial dysfunction and decreased oxidative stress normalized to normal levels. Telmisartan significantly improved basal endothelial function and decreased nitrotyrosine plasma level. In patients treated with telmisartan, a near-normalization of both flow-mediated vasodilation and oxidative stress was achieved when glycemia was normalized. Instead, adding vitamin C infusion did not show further effect on endothelial function or nitrotyrosine plasma levels [186]. A double-blind placebo-controlled clinical trial, conducted for 2 months and involving 35 patients with type 2 diabetes mellitus, has shown that lycopene, probably by increasing TAC and inhibiting MDA-LDL formation, may attenuate T cell-dependent adaptive (proatherogenic) immune

response. With enhancement of innate immunity and hence prevention of ox-LDL uptake by macrophage and foam cell formation, lycopene should be effective in prevention of long-term diabetic complications, notably cardiovascular disease [187]. One thousand two hundred and two people seen in dermatology clinics who did not have type 2 diabetes at baseline were studied in a randomized, double-blind, placebo-controlled trial to receive oral administration of Se (200 $\mu\text{g}/\text{d}$) or placebo. The average follow-up was 7.7 years. According to this study, Se supplementation does not seem to prevent type 2 diabetes, and it may increase the risk for the disease [188]. Vitamin C has a dose-dependent effect on the cellular contents of antioxidants and on vitamin E content of LDL in elderly patients with type 2 diabetes mellitus, but these changes are not sufficient to decrease the LDL susceptibility to peroxidation [189]. DEX (dextrothiamine: R-lipoic acid) therapy appears to reduce endothelial dysfunction in type 2 diabetes mellitus, especially in men with long history of type 2 diabetes mellitus and having poor glucose control (study involving 114 diabetic recipients). DEX is safe and well tolerated, and dyspepsia appears to be the most relevant side effect of DEX treatment [190]. Oxidative stress plays a key role in long-term β -cell dysfunction in type 2 diabetes. It has been shown that treatment with ALA improves functional outcomes, like insulin sensitivity in type 2 diabetic subjects [191]. Supplementation with vitamin E appears to lower plasma glucose in type 2 diabetic subjects [192], but 900 mg vitamin E/day lowers plasma glucose but may not improve pancreatic response to glucose. In humans no relationship between vitamin E intake and severity of retinopathy in type 2 diabetics has been observed [193, 194]. It has also been observed that there is no association between risk of retinopathy and intake of vitamins C and E from foods and supplements [195]. Plasma vitamin E-to-lipid ratio is lower in diabetic subjects than control subjects, and this effect is even more pronounced in diabetic subjects with neuropathy [196]. The same study has also shown that plasma vitamin E-to-lipid ratio is also inversely related to an assessed score of neuropathy, suggesting that diabetic subjects with neuropathy have higher levels of oxidative stress than those without this complication.

15.12 LIVER DISEASES

The liver is the largest organ in the body, weighing approximately 1.36 kg, and exerts several vital functions. It metabolizes substances present in blood, preparing them for excretion, synthesizes many essential proteins, produces bile, and regulates some key nutrients such as glucose, cholesterol, and amino acids. An increase in liver free radicals can lead to inflammation. Normally

the liver uses internally generated antioxidants to neutralize the toxin-derived free radicals, but when the liver antioxidants are low because of alcohol or chronic drug use damage from free radicals increases, resulting in inflammation and formation of scar tissue (fibrosis). Therefore it is important to maintain a constant supply of antioxidants and a healthy lifestyle (abstaining from all alcohol and avoiding environmental toxins) to reduce the strain on the liver. Alcohol lowers liver antioxidant levels, including vitamin E and S-adenosyl-L-methionine, making the liver vulnerable. In addition, alcohol lowers glutathione, an important internal antioxidant. Because heavy drinkers consume a substantial number of calories as alcohol, they consume less vitamin- and mineral-rich food, exacerbating alcohol-induced nutritional deficiencies that include low levels of vitamin C, riboflavin, zinc, pyridoxine (vitamin B6), and vitamins. A randomized clinical trial has compared antioxidant and corticosteroid treatments, showing that corticosteroids, in the form of prednisolone 30 mg daily, are superior to a broad antioxidant cocktail in treatment of severe alcoholic hepatitis [197]. A combination of seven different antioxidants administered orally (glycyrrhiza capsules, 500 mg, bid; schizandra capsules, 500 mg, tid; ascorbate capsules, 2000 mg, tid; L-glutathione capsules, 150 mg, bid; silymarin capsules, 250 mg, tid; lipoic acid capsules, 150 mg, bid; d- α -tocopherol, 800 IU/day) in patients with chronic hepatitis C virus (HCV) infection has been associated with a significant decline in ALT levels in 52% of patients versus 20% of patients who received placebo. Histology activity index (HAI) score at the end of treatment was reduced in 48% of patients versus 26% of patients who received placebo. HCV-RNA levels decreased by 1 log or more in 28% of patients who received antioxidant therapy versus 12% of patients who received placebo (not significant) [198]. Antioxidant therapy, alone or in combination with corticosteroids, does not improve 6-month survival in severe alcoholic hepatitis patients [199]. An analysis of nine randomized clinical trials including 434 patients with alcoholic liver diseases and the supplementation of SAME has shown no significant effects on all-cause mortality, liver-related mortality, liver transplantation, or complications [200].

15.13 PANCREATITIS

Acute pancreatitis (AP) is an acute inflammatory condition probably due to the activation of enzymes in the pancreatic acinar cells. Chronic pancreatitis (CP) is a progressive inflammatory disorder that is characterized by recurrent episodes of severe abdominal pain. There is evidence that the pathogenesis of both AP and CP can be associated with oxidative stress. In fact, it has been

observed that free radical activity and oxidative stress indices, such as lipid peroxide levels, are higher in AP or CP patients' blood and duodenal juice [201]. Treatment with Se, beta-carotene, L-methionine, vitamins C and E, or placebo for 10 weeks in patients with confirmed CP ($n=36$) has been associated with significant improvements in quality of life in terms of pain, physical and social functioning, and general health perception [202].

Allopurinol (300 mg), a hydroxyl radical scavenger, has been tested to determine a possible decrease in the rate of PEP, but it does not appear to reduce the overall risk of PEP [203]. Glutamine has been used in AP in combination with standard total parenteral nutrition (TPN; $n=28$), and a decrease in the duration of TPN therapy and hospitalization without a change in the total cost of parenteral feeding has been demonstrated [204]. Another similar study ($n=44$) has shown that even though TPN therapy containing glutamine reduces infectious morbidity, it has no significant effect on hospitalization and total mortality [205]. Both studies showed laboratory improvement in AP after administration of glutamine, such as an increase in serum albumin or decrease in CRP. Another study ($n=14$) has shown that glutamine supplementation does not significantly influence TNF- α or IL-6 release but does reduce median IL-8 release by day 7 in the glutamine group, while it increases in the conventional group [206]. Another nonblinded study examined the administration of glutamine in AP for 10 days starting either on the day of admission or 5 days after admission. Investigators reported an improvement in all clinical findings including hospitalization, infection, and mortality rate [207]. In contrast, another clinical trial ($n=78$) has shown that CP patients with chronic pain who were admitted to hospital and who received pethidine with or without allopurinol had a reduction in pain and gastric tenderness. Hospitalization also decreased in allopurinol-treated patients [208]. Another clinical study ($n=13$) has shown that 4-week allopurinol administration does not reduce pain in CP compared with placebo [209]. Allergy, general malaise, and gastrointestinal disturbances were adverse events of allopurinol. It has been shown that 10 g/day vitamin C decreases hospitalization and duration of disease and increases the cure rate in patients ($n=83$) with AP treated for 5 days. Proinflammatory cytokines and CRP were also diminished by vitamin C administration [210].

A combination of various antioxidants, including Se, beta-carotene, vitamin C, vitamin E, and methionine, administered to CP patients ($n=28$) has been shown to reduce the pain experienced in this condition [211]. Another study ($n=36$), using the same antioxidants at the same doses but with greater bioavailability in CP patients, has shown a reduction in pain after 10-week

combined antioxidant treatment. Quality of life, physical and social functioning, and health perception were also enhanced [202]. Another clinical trial ($n=147$) involving the use of the same antioxidant regimen, administered for 6 months, confirmed the previous trials, showing that pain and hospitalization were reduced [212]. The same combination of antioxidants, studied in 12 CP patients, has shown a reduction in pain and hospitalization [213], but some adverse effects, namely, headache, nausea, vomiting, and constipation have been reported. A pilot study involving 20 patients with CP who received 500 mg of curcumin with 5 mg of piperine or placebo for 6 weeks has shown a significant reduction in erythrocyte MDA levels following the curcumin therapy. A significant increase in glutathione (GSH) levels was also observed. There was no corresponding improvement in pain, and no adverse effects were reported [214].

N-acetylcysteine (NAC), a free radical scavenger that stimulates glutathione synthesis, has been tested in a trial involving 106 patients. NAC (600 mg) was given orally 24 h and 12 h before ERCP, and 600 mg was given intravenously twice a day for 2 days after ERCP. The rate of PEP was not significantly reduced. In addition, urine amylase activity, total bilirubin, alanine and aspartate aminotransferases, and white blood cells showed no change [215]. Another double-blind, placebo-controlled trial, involving 256 patients who received intravenous NAC at a loading dose of 70 mg/kg 2 hours before and 35 mg/kg at 4-hour intervals for a total of 24 hours after the procedure, has shown that there were no statistical differences in the incidence or severity of PEP grades between the groups. The mean duration of hospitalization for PEP was not different in the NAC group compared to the placebo group [216]. The results of those studies showed the absence of any beneficial effect of NAC on the incidence and severity of ERCP-induced PEP. A double-blind trial involving 321 patients who were given a single dose of natural beta-carotene 12 hours prior to the procedure has shown that the overall incidence of AP was not significantly different between the beta-carotene group and the placebo groups. The rate of severe PEP was lower in the beta-carotene-treated group [217]. SAME, a highly bioactive metabolite of methionine and a precursor of glutathione, has been investigated in two clinical trials investigating PEP. SAME did not enhance the clinical outcomes in either AP [218] or CP [219] patients. However, laboratory indices, such as free radical activity, were better after 10 weeks of SAME administration in CP patients. Two placebo-controlled clinical trials [220], examining a combined antioxidant therapy on recurrent CP, showed a significant decrease in pain and an elevation in serum antioxidant biomarkers. SAME has also been examined as an antioxidant, alone or in

combination with Se and beta-carotene, but proved to be ineffective in patients with recurrent PEP.

15.14 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic progressive autoimmune disorder characterized by symmetric erosive synovitis and destruction of the tissues within the joints leading to consequent physical disability. ROS has been correlated to the etiology of RA in an analysis of 20 randomized clinical trials (RCTs) [11 in inflammatory arthritis and 9 in osteoarthritis (OA); Se for RA ($n=5$); vitamin E for inflammatory arthritis ($n=5$); and vitamin E for OA ($n=7$)]. One study suggests the superiority of vitamin E over placebo, and three RCTs suggest equivalence between vitamin E and diclofenac in the treatment of inflammatory arthritis. In OA, four RCTs compared vitamin E with placebo. Two shorter-term studies have evidenced a positive effect of vitamin E in OA, while two longer-term studies did not. Two further RCTs suggest equivalence between vitamin E and diclofenac in the treatment of OA. An isolated positive result for vitamin C in OA is of doubtful clinical significance [221]. Antioxidants and cardiovascular disease (CVD) risk factors in participants with RA and non-RA control subjects have been studied, observing that plasma levels of antioxidants alpha-carotene, beta-cryptoxanthin, lutein/zeaxanthin, and lycopene were significantly lower in RA subjects compared with non-RA subjects. Compared with non-RA participants, RA subjects were more likely to have increased CRP levels [222].

15.15 KIDNEY DISEASES

Activated macrophages, vascular cells, and various glomerular cells are considered important ROS sources in kidneys. In particular, various diseases affecting the kidneys, namely, glomerulonephritis, tubulointerstitial nephritis, chronic renal failure, and IR injury, have been associated to ROS. Serum sulfite, sulfate, cysteine, homocysteine, cysteine sulfinic acid, and γ -glutamylcysteine are elevated in patients on hemodialysis, suggesting an accelerated catabolism of sulfur-containing amino acids, a reduced elimination of sulfite/sulfate, or both. It has been shown that dietary supplementation with vitamin E (400 mg/day for 6 months) in IgA nephropathy patients, a particular group of patients characterized by low vitamin E level and high oxidative stress, is able to reduce oxidative stress [223]. A study investigating the role of lovastatin or of hypolipemic diet on oxidative stress in hemodialyzed patients has shown that the level of 8-OHdG decreased considerably only in the

lovastatin-treated group and that the level of total antioxidant status (TAS) increased significantly in the lovastatin-treated group, decreased in the diet-treated group, and remained unchanged in the untreated group. Therefore lovastatin, but not hypolipemic diet alone, has an antioxidant effect in hemodialyzed patients, although the determinants of the antioxidant effect of statins in patients with chronic renal failure are unclear [224]. NAC has been studied in 30 uremic patients on hemodialysis (HD), suggesting that it could improve the ED by preventing the reduction of FMD in patients on HD [225]. Al-Awadi et al. [226] conducted an RCT to determine whether extracorporeal shock wave lithotripsy (ESWL) produces ischemia and reperfusion injury in kidneys and whether oral administration of antioxidants (2 capsules of Nature Made[®] antioxidants [each Nature Made[®] antioxidant capsule (Pharmavite Corporation, Mission Hills, CA, USA) contains high levels of Vitamin A (as beta-carotene) 10,000 IU, vitamin C 250 mg, and vitamin E 200 IU, and mineral supplements like zinc 7.5 mcg and Se 15 mcg] could protect from these complications. At 24 hours after ESWL, the patients who received antioxidants had significantly reduced mean MDA serum concentration, higher levels of serum ascorbic acid and serum albumin, lower α -tocopherol-to-cholesterol ratio, and lower urinary albumin and β_2 -microglobulin levels compared with patients who did not receive any antioxidants. These findings point out that treatment with ESWL generates free radicals through an ischemic-reperfusion injury mechanism and that oral administration of antioxidant may protect these patients from short-term renal injury caused by ESWL.

15.16 CONCLUDING REMARKS

This overview on antioxidant clinical use should be updated monthly because of the great number of contributions in this field. They produce evidence-based data on antioxidant benefit to prevent or counteract the ROS-induced damage in acute and chronic diseases as well as in surgical ischemia-reperfusion, surgical trauma, and so on. The choice of the right compound that will be effective to treat a specific disease is a very hard challenge and often a pool of natural or synthetic antioxidant molecules, contemporaneously administered, gives a benefit that cannot be achieved by a single molecule. From this perspective, antioxidant therapy must be considered a relevant support to restore physiological redox balance in several pathological conditions such as neurodegenerative diseases and in cancer prevention. Further insights into the multimodal pathogenesis of incurable illnesses will help, in the future, to

better define optimal protocols, in terms of scheduling, timing, and synergy with the emerging drugs (especially biologicals), in order to reduce their side effects and enhance their ability to heal.

15.17 CONFLICT OF INTEREST STATEMENT

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in this chapter.

15.18 STATEMENT OF AUTHORSHIP

The authors hereby certify that all work contained in this review is original work of Tommaso Iannitti and Beniamino Palmieri. The authors claim full responsibility for the contents of the article.

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THE PROTECTIVE ROLE OF GRAPE SEED POLYPHENOLS AGAINST OXIDATIVE STRESS IN TREATING NEURODEGENERATIVE DISEASES

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16.1 INTRODUCTION

Alzheimer disease (AD) is the most common type of dementia in the United States. Victims of AD commonly display a loss of memory, inability to learn new things, loss of language function, deranged perception of space, inability to do calculations, depression, delusions, and other cognitive deficiencies. AD is ultimately fatal within 5–10 years of its onset. It is estimated that approximately 5 million people in the United States currently have AD [1], with an estimated cost to society of more than \$100 billion per year. Up to 14 million people in the United States are projected to be affected by AD by the middle of this century if effective therapies are not developed [1]. To date, there is no cure for AD; thus researchers are continually exploring novel avenues for the prevention and treatment of this condition. It is important to note that even delaying AD onset by a few years would lead to significant reductions in disease prevalence and, consequently, its burden on health care systems. The few agents that are approved by the FDA for the treatment of AD have only modest efficacy in modifying clinical symptoms, and none appears to affect disease progression or prevention [2]. Historically, the majority of candidate therapeutics have been directed toward cholinergic neurons, which are especially vulnerable in AD and cognitive alterations [2, 3]. More recently, agents are being developed to interfere with the β -amyloid ($A\beta$) protein precursor pathway, which is thought to be

responsible for $A\beta$ -mediated neuronal dysfunction and death [4, 5].

16.2 ALZHEIMER DISEASE NEUROPATHOLOGY FEATURES: IMPLICATIONS FOR THERAPEUTIC DEVELOPMENTS

While the classification and diagnosis of AD are based on the cognitive behavior of an individual, the roots of the disease lie in the neurological pathology of its victims [6]. The two defining neuropathology features of AD are abnormal aggregation and deposition of $A\beta$ peptides and tau protein in the brain as, respectively, extracellular neuritic plaque (NP) and intracellular neurofibrillary tangles (NFT) [7].

$A\beta$ species with different amino and carboxyl termini are generated from the ubiquitously expressed amyloid precursor protein (APP) through sequential proteolysis by β - and “proamyloidogenic” γ -secretases [8, 4, 5]. A 40-amino acid form of $A\beta$ ($A\beta_{1-40}$) is the major secreted species. However, a 42-amino acid form of $A\beta$ ($A\beta_{1-42}$), which contains two additional residues at its carboxyl terminus, is thought to initiate AD pathogenesis [9]. $A\beta_{1-42}$ aggregates much more readily than $A\beta_{1-40}$ in vitro and is also deposited earlier and more consistently in AD brain. In humans, AD-causing mutations elevate plasma $A\beta_{1-42}$ levels by approximately 30–100% [10].

Even mutations showing small increases in A β 1-42 levels are associated with the onset of dementia in the fourth or fifth decade of life. In the Tg2576 transgenic mouse model of AD, the same mutations produce increases in A β 1-42 levels and markedly accelerate A β deposition [11]. In light of this evidence, a major effort from both academia and industry is presently focused on developing pharmacological strategies that would delay the initiation and/or slow the progression of AD-type A β neuropathology in the brain. Recent evidence from experimental AD mouse models indicates that accumulation of soluble high-molecular-weight oligomeric A β species in the brain, rather than deposition of NP *per se*, may be specifically related to spatial memory reference deficits. In particular, experimental evidence demonstrated that high-molecular-weight oligomeric A β species may disrupt hippocampal long-term potentiation and synaptic plasticity and induce synaptic deficits [12–15]. Consistent with the hypothesis that soluble high-molecular-weight oligomeric A β species play a key role in AD memory dysfunction, experimental therapeutic evidence demonstrated that neutralization of soluble high-molecular-weight A β oligomeric species from the brain causally improves spatial memory functions in a mouse model of AD [16].

Despite strong genetic data arguing that A β neuropathology is sufficient to cause AD [17], progressive cognitive decline and neuron and synapse loss in AD are best correlated with tau neuropathology [18]. In the AD brain, tau proteins, particularly hyperphosphorylated tau, are found aggregated into progressively larger polymeric species that are ultimately deposited as insoluble NFTs [19]. NFTs themselves are not necessarily the tau species inducing neurotoxicity, as evidence in experimental mouse models suggests that neuronal loss and memory impairment may occur before NFT formation in the brain [20, 21]. Instead, evidence indicates that accumulation of multimeric tau aggregates may play a more important role in AD memory dysfunction [21]. Consistent with this hypothesis, in transgenic mouse models levels of tau multimers are significantly correlated with memory index [21]. Moreover, neuronal loss and memory impairment in a mouse model of tauopathy can be mitigated by reducing tau expression without affecting the number of NFTs [20]. Aggregation of tau is a seed-nucleation process. Formations of hyperphosphorylated oligomers serve as nucleation sites that sequester additional hyperphosphorylated tau as well as normal nonphosphorylated tau [22]. Thus a predominant theory of tau-mediated neurodegeneration is based on a “toxic gain of function” model, in which abnormally phosphorylated tau promotes sequestration of both hyperphosphorylated and normal tau from microtubules, leading to microtubule instability and alteration

of microtubule-mediated processes, including abnormalities in axon transport among others [22].

These considerations strongly suggest that reducing the accumulation of soluble oligomeric A β peptides and tau species in the brain, as opposed to dissociating or preventing NP and/or NFT formation or deposition, may be a more productive approach to AD therapy. Conceptually, it might be possible to reduce brain accumulation of oligomeric tau species by reducing tau aggregation and/or by promoting tau clearance. Similarly, it might be possible to reduce oligomeric A β peptides in the brain by reducing A β generation, reducing A β aggregation, and/or promoting A β clearance. As discussed in more detail below, we recently demonstrated for the first time that dietary supplementation with red wines that is equivalent to moderate wine consumption in humans effectively attenuated the development of A β -mediated AD-type neuropathology and cognitive dysfunction in a mouse model of AD. Moreover, our evidence demonstrated the polyphenolics in the red wines as principle bioactive components that may modulate neuropathology A β phenotypes by reducing A β generation as well as A β aggregation.

16.3 POTENTIAL ROLES OF RED WINES AND WINE-DERIVED POLYPHENOLS IN ALZHEIMER DISEASE PREVENTION AND/OR THERAPY

While genetic factors are highly relevant in early-onset AD cases, their significance diminishes in late-onset sporadic AD cases, which is the most common form of AD [2]. Nongenetic factors, including modifiable lifestyle dietary regimens such as moderate consumption of alcoholic beverages, are receiving increasing attention in AD research, especially in light of the recent epidemiological studies indicating that moderate wine consumption may influence the relative risk for AD clinical dementia [23]. For example, while little is known about the beneficial role of red wine in AD dementia onset, recent studies suggested that the neuroprotective efficacy of red wine may be mediated, in part, by the antioxidant activities of polyphenols in the wine. Wine-derived polyphenols are known to have strong inhibitory effects on lipid peroxidation. Moreover, evidence indicates that several types of natural polyphenols may have neuroprotective effects both *in vivo* and *in vitro*, possibly through their abilities to scavenge reactive oxygen species, which may have profound implications for the overall protective effects of red wine in neurodegenerative disorders and stroke outcomes [24].

Presently, little is known about the potential role of red wine in AD. Recent prospective studies showed that moderate intake of red wine may decrease the relative

risk for AD [23]. We therefore explored whether red wines may beneficially modulate AD phenotypes in the Tg2576 AD mouse model.

16.4 THE Tg2576 AD MOUSE MODEL

Tg2576 mice are transgenic mice engineered to express a mutant form of the human APP harboring the Swedish [Lys670 → Asn, Met671 → Leu] mutation that is found in a subset of familial AD patients. Recapitulating select features of AD, the Tg2576 mouse model is characterized by progressive development of A β neuropathology and cognitive decline. Originally generated by Dr. Karen Hsiao [25], Tg2576 is the most commonly used animal model for studying the mechanisms underlying A β -mediated AD neuropathology and cognitive deterioration. This animal model is also commonly used for testing novel AD therapeutic strategies.

16.5 EXPLORING THE POTENTIAL BENEFITS OF MODERATE RED WINE CONSUMPTION IN Tg2576 MICE

In our initial studies, we treated Tg2576 mice with a dietary supplement of a red Cabernet Sauvignon wine to explore the impact of wine consumption on A β neuropathology and cognitive functions [26]. This was generated from *Vitis vinifera* by our collaborators at the University of Florida as previously described [27]. Our Cabernet Sauvignon contained ~12% alcohol, as determined by ebulliometry, and had a titratable acidity (as tartaric acid) of 6 g/l and a pH of 3.6 [26]. The wine was delivered to the mice by dilution into the drinking water to a final ethanol concentration of 6% [26]. In a parallel control study, Tg2576 mice were provided with drinking water containing 6% ethanol [26]. Wine (or ethanol control) treatment was initiated at 3 months of age before animals developed A β neuropathology or cognitive impairment, and continued to about 10.5 months of age when Tg2576 mice are typically characterized by moderate A β neuropathology and cognitive dysfunction.

In our Cabernet Sauvignon study, we observed that each mouse consumed ~4 ml of wine-adulterated water per day. We calculated that ~7% of the total daily energy consumption in Cabernet Sauvignon-treated Tg2576 mice was derived from wine. This is equivalent to wine-derived energy intake in the human following moderate wine consumption of one 5-oz glass of red wine for a woman (accounting for 6.2% of energy intake) and two 5-oz glasses of red wine for a man (accounting for 10% energy intake) [26]. We also used

an FDA-recommended criterion that incorporates body surface area for calculating equivalent drug doses across species [human equivalent dose in mg/kg = animal dose in mg/kg \times (animal wt in kg/human wt in kg)] [27] as an independent means of estimating human wine consumption that is equivalent to what mice received in our study. We calculated that Cabernet Sauvignon-treated mice consumed ~8 g of alcohol/kg body wt/day, which is equivalent to a human daily intake of 39.5 g of alcohol per day, or daily consumption of 2.3 5-oz glasses (329.2 ml) of Cabernet Sauvignon. Thus, on the basis of two independent calculations, we determined that mice in our study consumed an amount of Cabernet Sauvignon considered moderate by the U.S. Departments of Agriculture and Health and Human Services [26].

16.6 MODERATE CONSUMPTION OF A RED CABERNET SAUVIGNON WINE ATTENUATES AD-TYPE NEUROPATHOLOGY AND COGNITIVE DETERIORATION IN Tg2576 MICE

At the end of the Cabernet Sauvignon treatment, we assessed cognitive functions in Tg2576 mice with the Barnes maze protocol. We found that moderate consumption of Cabernet Sauvignon attenuated the onset of cognitive deterioration in the Tg2576 transgenic AD mouse model (Fig. 16.1A). In parallel control studies we found that moderate Cabernet Sauvignon consumption had no detectable impact on cognitive behavioral performance in age-, sex-, and strain-matched wild-type (WT) mice [26]. We found that cognitive benefits of wine treatment were associated with significant reduction of AD amyloid neuropathology in the brains of Tg2576 mice (Fig. 16.1B). Moreover, we found that Cabernet Sauvignon treatment might modulate A β neuropathology, in part, by promoting brain activity of α -secretase that interferes with generation by cleaving the APP within the A β sequence [26].

In light of evidence that natural polyphenols may exert neuroprotective effects, we explored whether polyphenolic components in Cabernet Sauvignon may be responsible, in part, for the efficacy of this red wine to modulate A β -mediated neuropathology responses. We extracted total polyphenolic components from the Cabernet Sauvignon with acetonitrile-butanol (1:1, v/v), followed by vacuum centrifugation, to concentrate the extracted polyphenol compounds and remove volatile organic components, including ethanol from the wine as well as organic solvents used in the extraction [26]. HPLC analysis confirmed that the contents of polyphenol components in the extract were comparable to Cabernet Sauvignon (data not shown).

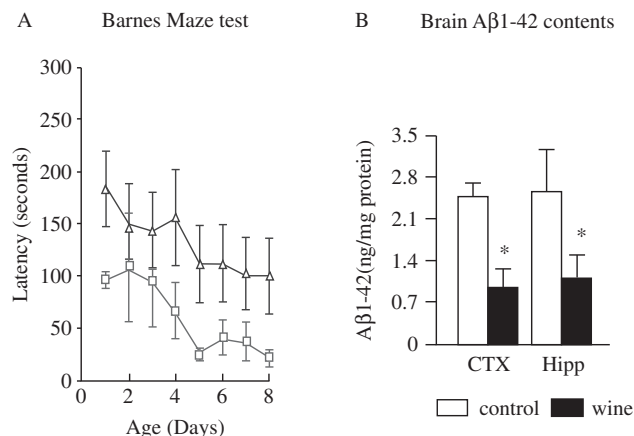


Fig. 16.1 Moderate consumption of Cabernet Sauvignon prevented cognitive impairment and attenuated Alzheimer disease (AD)-type neuropathology in the brains of Tg2576 mice. Tg2576 mice were treated with Cabernet Sauvignon by dilution of the wine into the drinking water. Control, nontreated mice were provided with unadulterated drinking water. (A) Spatial memory function assessment of Tg2576 mice treated with Cabernet Sauvignon at 10.5 months of age with the Barnes maze. Latency represents time (in seconds) to escape the platform, and points on the graph represent means (\pm SE). Statistical analysis by 2-way ANOVA: Cabernet Sauvignon group vs. control, 2-way ANOVA, $P < 0.0001$ for wine treatment, $P < 0.4379$ for escape latency over learning trials. (B) Assessment of A β 1-42 peptide concentrations in neocortex (CTX) and hippocampus (Hipp) of Cabernet Sauvignon-treated or control Tg2576 mice. Bar graphs represent group means \pm SE, $n = 6-9$ animals per group; * $P < 0.05$, 2-tailed Student's t -test.

We tested the efficacy of the Cabernet Sauvignon polyphenolic preparation to modulate the generation of A β peptides. For these studies, we prepared primary corticohippocampal neuron cultures derived from Tg2576 mice; these neuron cultures are known to generate A β peptides. We then treated primary corticohippocampal neuron cultures with the Cabernet Sauvignon polyphenolic preparation and measured A β generation by these neurons. Consistent with our observation in Cabernet Sauvignon-treated Tg2576 mice, we found that polyphenolics extracted from Cabernet Sauvignon significantly decrease generation of A β peptides by Tg2576 neuron cultures in a dose-dependent manner (Fig. 16.2). Similar to what we found in the brain of Cabernet Sauvignon treated Tg2576 mice, we also found that treatment with the Cabernet Sauvignon polyphenolic extract significantly reduced α -secretase activity in Tg2576 neuron cultures [26].

Collectively, observations from our in vivo studies on moderate consumption of the red Cabernet Sauvignon wine in Tg2576 AD mice and our in vitro mechanistic studies using Tg2576 corticohippocampal neuron cultures provide the first comprehensive experimental

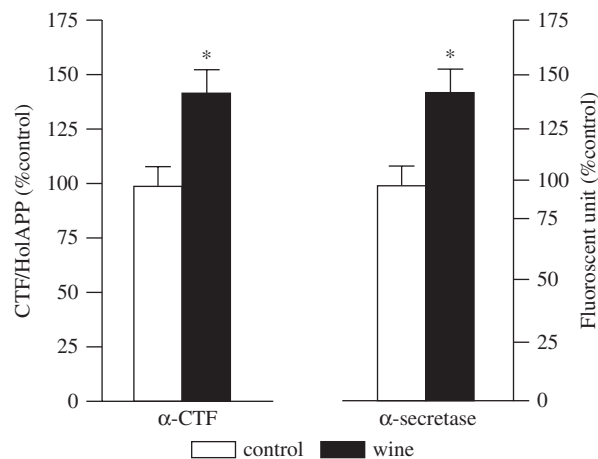


Fig. 16.2 Cabernet Sauvignon polyphenol extract promotes neuronal α -secretase activity. Primary corticohippocampal neuron cultures derived from Tg2576 mice were treated with 100 μ g/ml Cabernet Sauvignon extract followed by assessments of neuronal α -secretase activity using independent methodologies. (Left) Assessment of the contents of amyloid precursor protein α -carboxyl terminal fragment (α -CTF). Cleavage of amyloid precursor protein by α -secretase generates α -CTF. Therefore, neuronal α -CTF content directly reflects α -secretase activity. (Right) Fluorometric assessment of α -secretase enzymatic activities in primary neuron cultures treated with 100 μ g/ml Cabernet Sauvignon polyphenol extracts. Bar graphs represent group means \pm SE, $n = 3$ per group; * $P < 0.05$, 2-tailed Student's t -test.

evidence supporting the hypothesis that polyphenolic components from red wines might provide benefit disease-modifying activities in AD.

16.7 EXPLORING FOR POTENTIAL BENEFICIAL AD DISEASE-MODIFYING ACTIVITY IN A RED MUSCADINE WINE WITH DIFFERENT POLYPHENOLIC COMPOSITIONS COMPARED TO CABERNET SAUVIGNON

To gather insights on the specific polyphenolic component(s) in red wines that might exert beneficial AD-modifying activities in vivo, we continued to assess potential AD-modifying activity in another red wine, namely, a red muscadine wine, whose polyphenolic content we found is distinctly different from the polyphenolic contents of the Cabernet Sauvignon, which we demonstrated to benefit AD phenotypes (see below). This red muscadine wine was also generated by our collaborator at the University of Florida from *Vitis rotundifolia* grapes [27]. The muscadine wine contains approximately 12% alcohol (determined by ebulliometry) and is characterized by a titratable acidity (as tartaric acid) of 6.9 g/l and a pH of 3.00. The phenolic

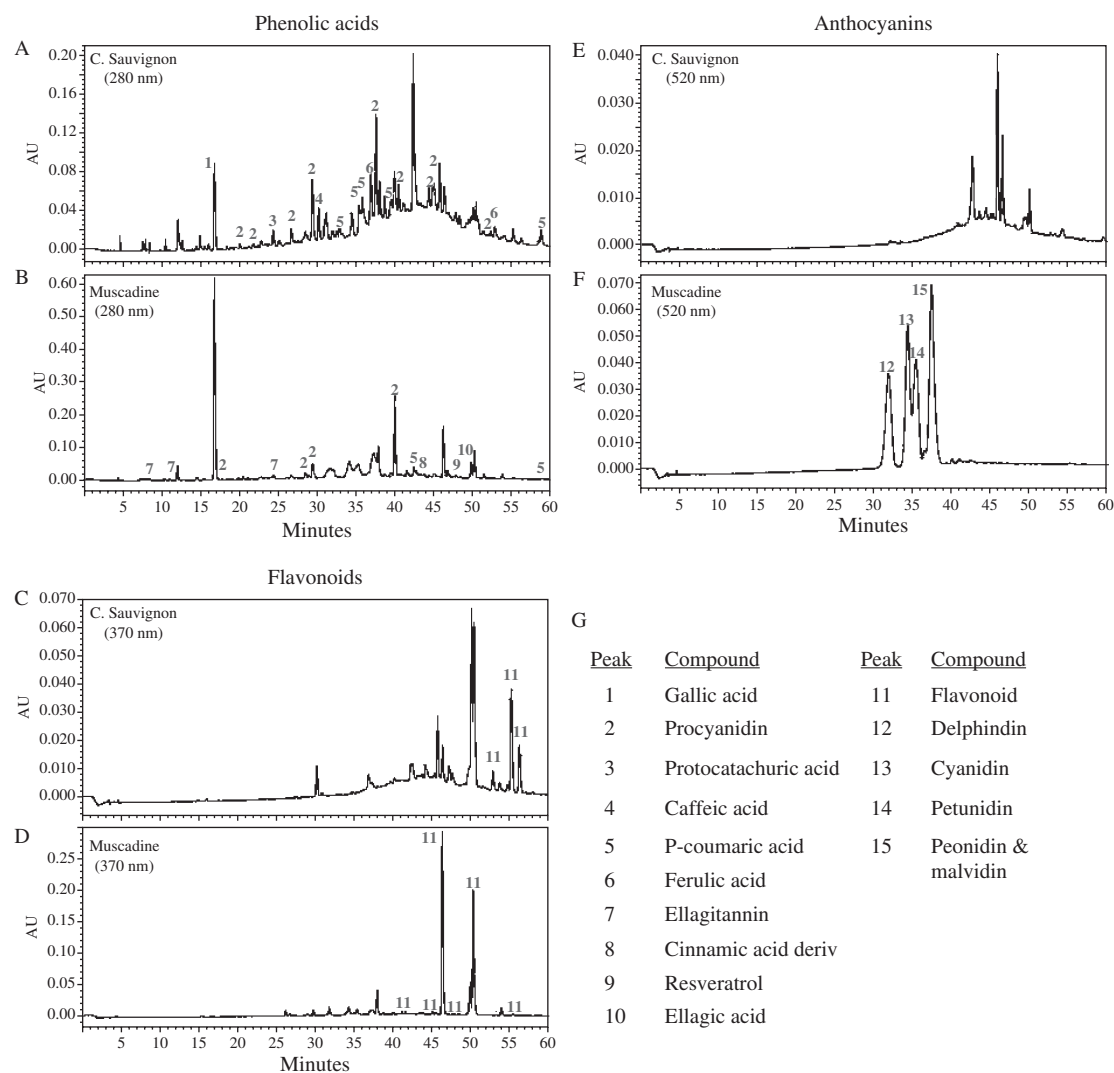


Fig. 16.3 Chemical analysis of Cabernet Sauvignon and muscadine wines. Constituent polyphenolic components in Cabernet Sauvignon (A,C,E) and muscadine (B,D,F) wines were analyzed by reverse phase HPLC using a C18 column. (A,B) Detection of phenolic acid compounds at 280 nm. (C,D) Detection of flavonoids at 370 nm. (E,F) Detection of anthocyanins at 520 nm. (G) Identification of polyphenols corresponding to peaks detected in panel (A-F) based on spectroscopic interpretations.

composition (as gallic acid and measured by the Folin-Coicalteau method) was 1.731 mg/l [27].

We analyzed chemical compositions of muscadine and Cabernet Sauvignon by reverse-phase chromatography using HPLC and an octadecyl silane column [29]. Different classes of polyphenolic compounds from the wines were detected at 280 nm (phenolic acids) (Fig. 16.3A,B), 370 nm (flavonoids) (Fig. 16.3C,D), and 520 nm (anthocyanins) (Fig. 16.3E,F), with select compounds identified based on spectroscopic interpretations from 200-600 nm (Fig. 16.3G) [29]. Our analysis demonstrated that the two wines are characterized by distinct component compositions of phenolic acid (Fig. 16.3A, B), flavonoid (Fig. 16.3C,D), and anthocyanin polyenolic compounds (Fig. 16.3E,F).

16.8 MODERATE CONSUMPTION OF RED MUSCADINE WINE ATTENUATES AD-TYPE NEUROPATHOLOGY AND COGNITIVE DETERIORATION IN Tg2576 MICE

We treated Tg2576 mice with the red muscadine wine at a dosage equivalent to moderate wine consumption, using a procedure comparable to that we used with our Cabernet Sauvignon studies [29]. Similar to our observation with Cabernet Sauvignon, we found that moderate consumption of the muscadine wine significantly attenuated the development of spatial memory decline (Fig. 16.4A,B) and AD-type A β neuropathology (Fig. 16.4C) in the Tg2576 AD mouse model. However, in contrast to our previous observation with Cabernet

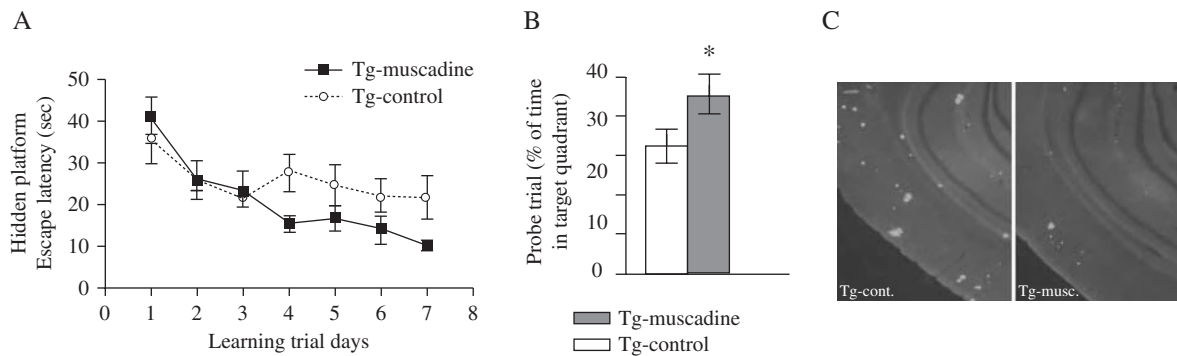


Fig. 16.4 Muscadine treatment improves spatial memory function and A β neuropathology in Tg2576 mice. (A,B) Assessments of spatial memory behavioral functions of 14 month old muscadine-treated (Tg-muscadine) and control, gender- and age-matched non-treated (Tg-control) Tg2576 mice using the Morris water maze protocol. (A) Learning trial hidden-platform acquisition curves. Tg-muscadine group performed significantly better than the control, non-treated group (Tg-control) [2-way ANOVA analysis of Tg-muscadine vs. Tg-control groups for muscadine treatment ($p < 0.05$, $F = 4.24$, $DFn = 1$, $DFd = 84$) and for training days ($p < 0.05$, $F = 6.43$, $DFn = 6$, $DFd = 84$)]. (B) Probe trial conducted 24 hours after completion of hidden-platform training. Muscadine-treated Tg2576 mice exhibited a significantly higher preference for the target platform compared to control, non-treated Tg2576 mice ($p < 0.05$, 2-tailed Student t test). In (A,B) Values represent group mean (+SEM); $n = 7$ –9 mice per group. (C) Assessments of A β neuropathology reflected by amyloid neuritic plaque density in cerebral cortex and in the hippocampal formation of brain specimens from muscadine-treated and control, non-treated Tg2576 mice. Representative micrograph of brain specimen stained for amyloid neuritic plaques in muscadine-treated (Tg-musc.) or in control, non-treated (Tg-cont.) Tg2576 mice. (See color insert.)

Sauvignon [26], we found that muscadine treatment did not modulate α -secretase activity or activities of other enzymes known to be involved in generation of A β peptides in the brain from APP [29]. Instead, we found that muscadine treatment reduced the accumulation of soluble high-molecular-weight oligomeric A β species in the brain, suggesting that muscadine treatment interferes with A β oligomerization (Fig. 16.5A). In parallel studies, we confirmed the efficacy of muscadine polyphenolics to interfere with the assembly of A β peptides into neurotoxic, high-molecular-weight oligomeric species (Fig. 16.5B). As illustrated by using a gel electrophoresis to assess steady-state A β aggregates, we demonstrated that polyphenolic components from the muscadine wine potently interfere with the formation of a synthetic A β peptide into high-molecular-weight aggregates (Fig. 16.5B). Collectively, our in vivo and in vitro evidence suggests that muscadine polyphenol may have benefited AD phenotypes in the Tg2576 mouse model of the disease by inhibiting the assembly of A β peptides into neurotoxic high-molecular-weight aggregates.

16.9 DIETARY GRAPE-DERIVED BIOACTIVE POLYPHENOLIC COMPONENTS: IMPLICATIONS IN AD THERAPY AND PREVENTION

The evidence from our studies with two independent red wines summarized above strongly supports the hypothesis that moderate red wine consumption might provide preventive and/or therapeutic value in AD. The

potential health benefits of wine consumption are generally ascribed to the polyphenol compounds that are present in high abundance, particularly in red wines [30, 31]. Since many of the wine-derived polyphenols are strong antioxidants, it is thought that red wine polyphenols may benefit AD (and other neurodegenerative disorders) by reducing the content of reactive oxygen species in the brain [32]. Aside from potential antioxidant activities, our accumulating preclinical evidence suggests that red wine polyphenols may also benefit AD by directly modulating A β -related mechanisms in the brain. Results from our studies demonstrated that polyphenolic components from the Cabernet Sauvignon wine may protect against the onset of AD-type A β -neuropathology and cognitive deterioration by promoting α -secretase activity in the brain. In contrast, our studies suggest that polyphenolic compounds from a red muscadine wine may modulate AD phenotypes by interfering with aggregation of A β peptides into neurotoxic high-molecular-weight oligomeric A β species in the brain. Because the two red wines are characterized by distinct polyphenolic component compositions, our evidence suggests that selective bioactive polyphenolic compounds may be responsible for A β -lowering and anti-A β aggregation activities. More studies will be necessary to identify and characterize specific polyphenolic components from red wines capable of exerting A β -lowering and/or anti-A β aggregation activity in the brain. Collectively, our evidence suggests the possibility of developing a “combination” of dietary polyphenolic compounds for AD prevention and/or therapy by modulating multiple A β -related mechanisms (Fig. 16.6).

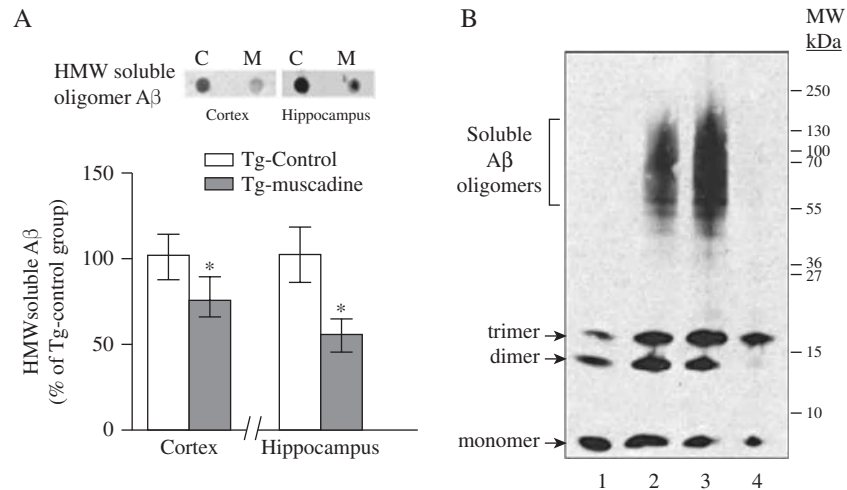


Fig. 16.5 Muscadine treatment reduces aggregation of A β peptides into neurotoxic high molecular weight aggregates. (A) Muscadine treatment significantly attenuated the accumulation soluble high molecular weight A β species in the brain of Tg2576 mice. The contents of soluble high molecular weight A β oligomeric species in the cerebral cortex and hippocampal formation of muscadine-treated or control, non-treated 14 month old Tg2576 mice were assessed by an immunological dot-blot (A, inset) Representative A11-immunoreactive dot-blot analysis of cortical and hippocampal formation brain specimens. Bar graphs represent means \pm SEM., $n = 6-8$ per group; * $P < 0.05$ vs. non-treated control Tg2576 group (2-tailed Student's t test). (B) Muscadine wine interferes with aggregation of synthetic A β 1-42 peptides into high molecular weight oligomer A β species, *in vitro*. Synthetic A β 1-42 peptides were aggregated in the absence or in the presence of muscadine wine. A β species were then resolved by molecular size, transblotted onto a nitrocellulose membrane, followed by immunodetection of A β peptides using and the 6E10 antibody. Lane 1 represents non-aggregated A β 1-42 peptides; Lane 2, aggregated A β 1-42 peptides; Lane 3, A β 1-42 peptides aggregated in the presence of 1.2% ethanol (the same amount of ethanol presented in the aggregation assay in the presence of muscadine in Lane 4); Lane 4, A β 1-42 peptides aggregated in the presence of 1 μ l muscadine wine. In this assay, aggregation of A β without in the absence or presence of ethanol in lanes 2 and 3, respectively, lead to generation of high molecular weight A β species that migrated slowly in the assay. In contract, addition of muscadine completely eliminate the generation of high molecular weight A β aggregates.

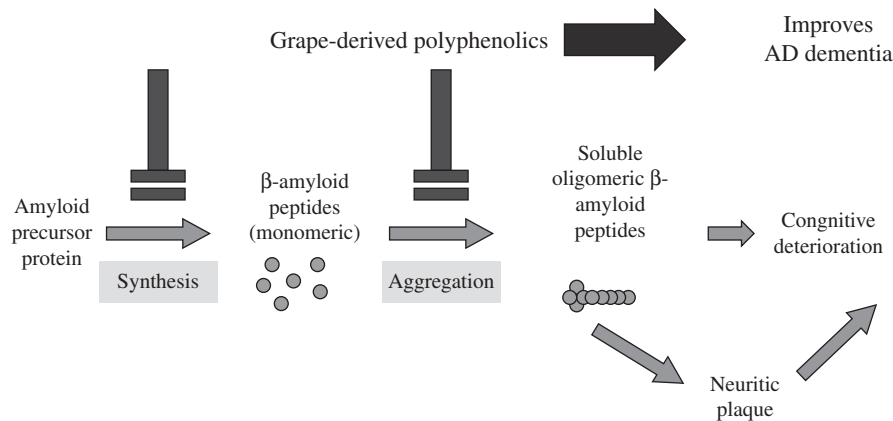


Fig. 16.6 Schematic of how grape-derived polyphenolics may benefit AD by modulating A β -mediated mechanisms. A β peptides are generated from amyloid precursor protein. Thereafter, monomeric A β peptides can be assembled into neurotoxic, soluble high-molecular-weight aggregates that may directly induce neuronal dysfunction that leads to cognitive deterioration. Further aggregation of soluble high-molecular-weight A β aggregates leads to deposition of insoluble A β aggregates as neurotic plaque in the brain that also induces neuronal dysfunction, in part, by promoting brain inflammatory responses. Bioactive polyphenols from grape-derived polyphenolics from red wine or other dietary products may improve interfere with A β -mediated pathological mechanisms by interfering with the generation (synthesis) of A β peptides from amyloid precursor protein and/or by interfering with assembly (aggregation) of A β peptides into neurotoxic, soluble high-molecular-weight species. This raises the possibility of improving AD dementia with a “combination” of dietary bioactive polyphenolic compounds.

In addition to the Cabernet Sauvignon and the muscadine wine, we recently demonstrated that other grape-derived products, namely, a grape seed polyphenolic extract [33] and a purple grape juice (Ho et al., unpublished observation), also exert bioactivity at the organism level and significantly interfere with the development of A β -related phenotypes in AD mouse models. In light of our observation that multiple dietary grape products with distinct polyphenolic component composition effectively protect against the onset and progression of AD phenotypes, we hypothesize that additional grape-derived products, including other red wines, might also provide beneficial disease-modifying activities in AD.

There is an urgent need for additional studies to identify specific bioactive polyphenolics from red wines or other grape-derived dietary products and to characterize the mechanisms of action of these bioactive polyphenolics. Information generated will provide the rational basis for developing selective bioactive dietary polyphenol(s) as lead compounds for clinical testing in AD. Moreover, this information will promote selection of food sources enriched in targeted bioactive polyphenols that ultimately could be incorporated as key components in the development of potential dietary guidelines for AD prevention and/or management.

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PHARMACOLOGICAL AND THERAPEUTIC PROPERTIES OF PROPOLIS (BEE GLUE)

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17.1 INTRODUCTION

Propolis has been used by humans for thousands of years and recently has enjoyed a boom in popularity. Bees have used propolis for millions of years, and humans have used it for thousands. Both species find it immensely useful and beneficial. Much of the bees' success in surviving through the ages may be accredited to propolis. The Greek physician Hippocrates prescribed the use of propolis to help heal internal and external sores and ulcers. Ancient Egyptians depicted propolis-making bees on vases and other ornaments and used the resinous substance to alleviate many ailments. Pliny, the Roman scholar, wrote much on the use of resins such as propolis in his massive book, *Natural History*. He touted the abilities of propolis to reduce swelling, soothe pain, and heal sores, to name a few.

In the *History of Plants*, written by John Gerard in 1597, propolis was noted for its ability to provide swift and effective healing for many conditions. During this era, propolis was used in many different healing ointments. Although propolis is vitally important to the colony, there are usually just a few propolis-gathering specialists in the hive. The bees gather propolis, sometimes called "bee glue," and carry it home in their pollen baskets. There they are met by one or two other worker bees that help them unload. These workers take the resinous material and add salivary secretions and wax flakes to it and then use the new product for numerous protective purposes as bee propolis. The bees use it to

coat the inside of the hive, including the passageway and the brood chambers. Propolis protects the hive in two ways: First, it reinforces the hive itself; second, it protects the hive from bacterial and viral infection. And it is these latter properties that humans have found so helpful through the centuries.

17.2 PROPOLIS

Propolis is a resinous yellow-brown to dark brown substance collected by worker honeybees from the growing parts of trees and shrubs (e.g., leaf buds, trunk wounds). The bees pack the propolis on their hind legs, and carry it back to their colony, where it is combined with beeswax and used by worker "hive" bees as a sealant and sterilant in the colony nest. These uses take advantage of the antibacterial and antifungal effects of propolis in protecting the colony against disease [1].

Propolis changes consistency with temperature. At temperatures below 15°C it is hard and brittle, but it becomes more pliable and sticky at higher temperatures. Propolis generally melts at 60–70°C, although some samples have been found to have a melting point as high as 100°C [2]. Propolis is collected by commercial beekeepers, either by scraping the substance from wooden hive parts or by using specially constructed collection mats. The raw product undergoes secondary processing to remove beeswax and other impurities before being

used in a variety of natural health care products (e.g., lozenges, tinctures, ointments, drinks).

17.2.1 History

Propolis, bee glue, a gum gathered by bees from various plants, has been used by humans since early times for various purposes, and especially as a medicine because of its antimicrobial properties [3]. Ancient Greek texts refer to the substance as a “cure for bruises and suppurating sore,” and in Rome propolis was used by physicians in making poultices. The Hebrew word for propolis is *tzori*, and the therapeutic properties of *tzori* are mentioned throughout the Old Testament. Records from twelfth-century Europe describe medical preparations using propolis for the treatment of mouth and throat infections and dental care [4].

One of the nonmedical uses of propolis is as a varnish, and it has been suggested that the special properties of Stradivarius violins may be partly due to the type of propolis used, although the claim cannot be substantiated.

17.2.2 Composition

At least 180 different compounds have been identified so far in propolis. The class of compounds present in propolis includes resins, waxes and fatty acids, essential oils, pollen, and minerals. The resins comprise flavonoids, phenolic acids, and esters (45–55%) whereas the essential oil contain 10% volatiles. There are nearly 12 free amino acids in pollen and 14 trace minerals, iron and zinc being the most common. The other organics present are ketones, lactones, quinines, steroids, benzoic acid, vitamins, and sugars. The most important pharmacologically active constituents in propolis are the flavones, flavonols, and flavanones (collectively called flavonoids) and various phenolics and aromatics. Flavonoids play a major role in plant pigmentation. However, the flavonoids present in propolis are not glycosides (they do not have sugar molecules attached to their chemical structure). The majority of flavonoids found in plants are glycosides.

Flavonoids are thought to account for much of the biological activity in propolis [5], although other phenolic compounds are also involved. At least 38 flavonoids have been found in propolis, including galangin, kaempferol, quercetin, pinocembrin, pinostrobin, and pinobanksin [6]. Other phenolics include cinnamic alcohol, cinnamic acid, vanillin, benzyl alcohol, benzoic acid, and caffeic and ferulic acid. The chemical composition of propolis is highly variable because of the broad range of plants visited by honeybees when collecting the substance [7].

At least 67 plant species have been reported to provide propolis material. Important sources include poplars, alders and birches, chestnut, ash, and willows. Variations in the beeswax content of raw propolis also affect the chemical composition. Studies indicate that the plant resins collected by bees are at least partially altered by honeybees before use in the hive.

17.3 HUMAN NUTRITION

Propolis also has nutritive value, because of the presence of small amounts of proteins, amino acids, minerals, and sugars. Vitamins include small amounts of A, B₁, B₆, C, and E [8]. Dihydroflavonoids, like those found in propolis, have been shown to aid the human body in absorbing vitamin C [9]. Propolis and a number of its components exhibit a wide variety of biological and pharmacological activities [6].

17.4 THERAPEUTIC PROPERTIES

Because of its strong antimicrobial activity, propolis is often known as a “natural antibiotic.” Propolis is reported to possess anticancer, apoptotic, wound healing, antiinflammatory, antiulcer, antiviral, anesthetic, and immunomodulatory properties.

17.4.1 Antimicrobial Properties

A large number of studies have shown an inhibitory effect on a variety of microorganisms (Table 17.1). Active components of propolis showing an antibacterial effect include pinocembrin, galangin, caffeic acid, and ferulic acid, whereas antifungal components include pinocembrin, pinobanksin, caffeic acid, benzyl ester, sakuranetin, and pterostilbene and antiviral components include caffeine. Propolis has been found to inhibit the synthesis of protein by bacteria, which may account for at least some of its antimicrobial effects [16].

17.4.2 Synergistic Properties

Most studies on the therapeutic properties of propolis have centered on the phenolic constituents (flavonoids and other phenolic compounds such as caffeic acid esters). Research has tended to isolate and test single substances in propolis. However, it is likely that the presence of a large number of compounds in propolis may produce a synergistic effect greater than the sum of the effects of individual components [31]. Studies have shown that the flavonoids in propolis exert significant antimicrobial activity compared to whole product extracts.

Propolis has also been shown to have a synergistic effect with certain antibiotics and to increase their effectiveness on some bacteria and yeasts, in some cases 100-fold [32]. Antibiotic-resistant strains of *Staphylococcus* were found to become sensitive to antibiotics in combination with propolis [33].

17.4.3 Anticancer Properties

Ethanol extracts of propolis have been found to transform human hepatic and uterine carcinoma cells in vitro and to inhibit their growth [34]. Substances isolated from propolis that produce this cytotoxic effect are quercetin, caffeic acid, and clerodane diterpendoid. Clerodane diterpendoid shows a selective toxicity to tumor cells.

Propolis was also found to have a cytotoxic and cytostatic effect in vitro against hamster ovary cancer cells and sarcoma-type tumors in mice [35]. The substance has also displayed cytotoxicity on cultures of human and animal tumor cells, including breast carcinoma, melanoma, colon, and renal carcinoma cell lines [36]. The component producing these effects was identified as caffeic acid, phenethyl ester.

Caffeic acid esters have been shown to inhibit chemically induced tumor production in mice, as well as having selective toxic effect on cells affected by genes that promote the development of cancerous cells [37].

A substance called artepillin C has been isolated from propolis, and it has been shown to have a cytotoxic effect on human gastric carcinoma cells, human lung cancer cells, and mouse colon carcinoma cells in vitro [38].

17.4.4 Apoptotic Characteristics

It is known that propolis ethanolic extract (100 µg/ml) causes apoptotic-like cell demise. Chemotherapy based on propolis, alone or in combination with vinorelbine, has been suggested to be a useful tool in prostate cancer therapy.

17.4.4.1 Treatment of Breast and Prostate Cancers.

There are mainly three types of propolis whose major anticancer ingredients are entirely different: (1) CAPE (caffeic acid phenethyl ester)-based propolis in Europe, the Far East, and New Zealand; (2) artepillin C (ARC)-based Brazilian green propolis; and (3) Brazilian red propolis. Neurofibromatosis (NF)-associated tumors require the kinase PAK1 for their growth, and CAPE-based propolis extracts such as Bio 30 suppress completely the growth of NF tumors in vivo by blocking PAK1 signaling. It was demonstrated that ARC suppresses angiogenesis, suggesting the possibility that ARC also blocks oncogenic PAK1 signaling. The study suggested that both CAPE-based and ARC-based propolis extracts are natural anti-PAK1 remedies and could be among the first effective NF therapeutics available on the market. Since more than 70% of human cancers such as breast and prostate cancers require the kinase PAK1 for their growth, it is quite possible that GPE could be potentially useful for the treatment of these cancers, as is Bio 30 [39].

Vinorelbine bitartrate, a drug widely used in prostate cancer therapy, was utilized as a reference drug because it is known to induce apoptosis [40]. The combined treatments of micronutrients viz. propolis extract and vinorelbine have been studied to test a possible vinorelbine dose reduction, avoiding its side effects without altering its cytotoxic action. Here, SEM and TEM analyses have also been performed to examine the morphological modifications induced; the observations confirmed apoptotic modifications after propolis treatment. They also measured cell cycle progression to study a correlation with p21 and p53, two well-known cell cycle checkpoints. The levels of HSP27 and HSP70, two chaperones exerting antioxidant/antiapoptotic functions, were also analyzed. The data indicated that

TABLE 17.1 Antimicrobial effects

Microorganisms	Targeted Action	Reference
<i>Bacillus larvae</i>	Destroyed	1
<i>Bacillus subtilis</i>	Destroyed	10
<i>Helicobacter pylori</i>	Inhibited	11
Methicillin-resistant <i>Staphylococcus aureus</i>	Inhibited	5
<i>Mycobacterium tuberculosis</i>	Inhibited	12
<i>Bacterioides nodosus</i>	Reduced foot rot	21
<i>Escherichia coli</i>	Inhibited	16
<i>Giardia lamblia</i>	Positive effect	20
<i>Klebsiella pneumonia</i>	Positive effect	22
<i>Staphylococcus</i> sp.	Inhibited	13
<i>Staphylococcus aureus</i>	Synergistic effect	14
<i>Streptococcus</i> sp.	Inhibited	15
<i>Streptomyces</i>	Inhibited	16
<i>S. sobrinus, mutans, cricetus</i>	Dental caries	17
<i>Saccharomyces cerevisiae</i>	Brewer's yeast	18
<i>Salmonella</i>	Potentially treated	19
<i>Aspergillus niger</i>	Positive effect	24
<i>Ascosphaera apis</i>	Inhibited	26
<i>Botrytis cinerea</i>	Fungicidal	25
<i>Candida albicans</i>	Synergistic effect	23
Herpes virus	Inhibited	27
Influenza (in mice) virus	Reduced mortality	29
Newcastle disease virus	Affected virus reproduction	30
Potato virus	Effective	28

propolis modulated cell cycle distribution, increasing p53 levels, without the induced HSPs being able to rescue DU145 from death. Hence chemotherapy based on propolis, alone or in combination with vinorelbine, as a potential useful tool for prostate cancer therapy as it effects increase in cell cycle control and the modulation of HSPs expression, reinforced this suggestion.

17.4.4.2 *Propolis-Induced Apoptosis of Carcinoma Cells*

The treatment of laryngeal cancer is comprehensive, based on surgery. Chemotherapy is an important component of the treatment for laryngeal cancer, which lacks ideal selectivity. The anticancer active ingredients contained in propolis have been identified as flavonoids, terpenes, sugars, esters, and other compounds, such as a natural combination, their mutual coordination role, given the anticancer effect of propolis [41]. Experimental results show that propolis inhibited chemical carcinogen-induced mutations [42]. Because radiotherapy and chemotherapy kill tumor cells, these would also damage the body's immune and hematopoietic cells, and therefore strengthening the immunity against radiation and chemotherapy-induced side effects, is the important task of cancer prevention and treatment. The results show that propolis on Hep-2 cells significantly inhibited cell proliferation and can induce apoptosis and the cell cycle to a certain extent. It can affect the cell cycle G₁ phase to S phase transition, but also affect the S phase of the transition to the G₂/M leading to tumor cell apoptosis and cell cycle non-specific blocking the whole process. With increase of the concentration of propolis, the killing effect on cancer cells was also enhanced. The study revealed that propolis inhibited the growth of laryngeal cancer cells, and the inhibitory action was through the mechanism of apoptosis [43–45].

In a study two new prenyl flavanones, propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced cytotoxicity in human melanoma A2058 cells and showed a strong capability to scavenge free radicals. In this study, propolin A effectively induced a cytotoxic effect on five different cancer cell lines. The levels of procaspase-8, Bid, procaspase-3, DFF45, and PARP were decreased in dose- and time course-dependent manners. Propolin A and propolin B were also capable of releasing cytochrome *c* from mitochondria to cytosol. The findings suggest that propolin A and propolin B may activate a mitochondria-mediated apoptosis pathway. All these results indicated that propolin A and propolin B may trigger apoptosis of A2058 cells through mitochondria-dependent pathways and also showed that propolin A and propolin B were strong antioxidants [46].

It was demonstrated that the prenyl flavanones propolin A and propolin B, isolated and characterized from Taiwanese propolis, induced apoptosis in human

melanoma cells and significantly inhibited xanthine oxidase activity. Furthermore, it the isolation of a third compound called propolin C was reported [47]. The chemical structure of propolin C has been characterized by NMR and HRMS spectra and was identical to nymphaeol-A. Propolin C was found to effectively induce cytotoxic effect on human melanoma cells, with an IC₅₀ of about 8.5 μM. In a study to address the mechanism of the apoptosis effect of propolin C, the effect of propolin C on induction of apoptosis-related proteins in human melanoma cells was evaluated. The findings suggested that propolin C may activate a mitochondrion-mediated apoptosis pathway [47].

17.4.5 Antioxidant Properties

The flavonoids concentrated in propolis are powerful antioxidants. Antioxidants have been shown to be capable of scavenging free radicals and thereby protecting lipids and other compounds such as vitamin C from being oxidized or destroyed. The polyphenolic-rich extracts of beeswax have been reported to exhibit antioxidant property in vivo [48–50]. In this study, the extracts were able to reduce CCl₄- and paracetamol-induced oxidative stress in rats, as evidenced by the changes in hepatic antioxidant and detoxifying enzymes.

Propolin C is also a potential antioxidant agent and shows a strong capability to scavenge free radicals and inhibit on xanthine oxidase activity with IC₅₀ of about 17.0 μM [47].

17.4.6 Wound Healing Properties

Propolis has been shown to stimulate various enzyme systems, cell metabolism, circulation, and collagen formation, as well as improving the healing of burn wounds [51, 52]. These effects have been shown to be the result of the presence of arginine in propolis [53]. Patients with tibial skin ulcers, aged from 23 to 98 years, were treated with propolis tincture in an ointment. The ointment was applied daily to the ulcerated area, which was also treated on the periphery with antibiotic ointments [54]. The treatment lasted for 4–12 weeks. At the end of treatment, 19 of the 84 treated patients exhibited no clinical signs of the condition, and 19 exhibited an improved condition [54].

Propolis was used in a trial of hospital patients with infected wounds. The propolis improved wound healing rates, while at the same time reducing infection. Over half of infective bacteria were eliminated within 4 days. Propolis did not produce antibiotic-resistant strains of the bacteria [55]. A study of topical application of propolis on wounds, burns, and ulcers showed up to an 80% increase in healing rate compared to control subjects using routine healing regimes.

Patients (229) with burns, clean wounds, infected wounds, or abscesses/ulcers were treated with cream containing propolis at two concentrations (2% and 8%). The higher concentration caused local intolerance in 18% of patients by day 9, whereas the lower concentration caused symptoms in only 1.8% of patients by day 16. Burns and wounds treated with the low-concentration cream healed in 11 days on average, septic wounds in 17.5 days, and 67% of ulcers in 38 days [56].

17.4.7 Antiulcer Properties

In a study to treat ulcerative colitis and Crohn disease by propolis in a double-blind clinical trial in Denmark, improvement was noted in patients with colitis, but no effect was shown against Crohn disease. Propolis has been shown to inhibit the development of externally induced stomach ulcers in rats. Flavonoid components of propolis have also been shown to have this effect. [57].

In a study conducted in patients (138) suffering giardiasis were treated with propolis extracts (10–20%). In children, 52% showed a cure at the lower dose. In adults, the cure rate was the same as for tinidazole, an antiprotozoan drug, at the 20% extract and 60% versus 40% for Undazole at a higher concentration (30% propolis extract) [58]. The efficacy of bee propolis in the treatment of acute and chronic colitis was investigated elsewhere and promising results were reported.

17.4.8 Skin Infection Effects

Propolis has been shown to be effective in inhibiting the growth of yeasts and fungi responsible for such skin infections as ringworm and athlete's foot. Propolis compounds showing activity against these organisms are the flavonoids and caffeic acid derivatives.

Clinical applications of propolis (1–10%) in ether or alcohol were effective against 10 superficial fungi and 9 deep-growing fungi. On oral treatment of 160 psoriasis patients with 0.3 g propolis 3 times daily for 3 months, about one-third were cured or greatly improved. Patients (110) infected with ringworm were treated with 50% propolis as an unguent. In 97 patients it was found to produce excellent results [59].

17.4.9 Antiinflammatory Properties

Studies on mice have shown that extracts of propolis have an antiinflammatory effect similar to that of indomethacin, a common drug used to treat inflammation. Again, flavonoids and caffeic acid are known to play a role in inhibiting the inflammatory response. Injections of an aqueous solution of propolis were used in the treatment of 22 patients with hip joint disease caused by aseptic necrosis of the thigh bone. A further 32 patients with the

same condition were given different forms of routine treatment. Significant improvement was observed in the patients given propolis. Patients (90) with cases of vagina and uterine cervix inflammation caused by pyrogens were treated with 3% propolis ethanol extract. Over 50% of the cases responded well to this treatment [60].

17.4.10 Anesthetic Properties

Propolis and some of its components produce anesthesia, which in some studies has been shown to be three times as powerful as cocaine and 52 times as powerful as procaine when tested in rabbit cornea. The anesthetic effect has been shown to be produced by pinostrobin, caffeic acid ester components in propolis [61]. The anesthetic effect may explain why propolis has been used for centuries in the treatment of sore throats and mouth sores. An anesthetizing ointment for dentistry using propolis has been patented in Europe [62].

17.4.11 Immunomodulatory Properties

Propolis has been shown to stimulate an immune response in mice [63]. More recently, Japanese researchers have shown that an extract of propolis produces a macrophage activation phenomenon related to the immune function in humans [64]. Propolis activates immune cells, which produce cytokines. The results help to explain the antitumor effect produced by propolis. Propolis has been shown to stimulate antibody formation in immunized mice. In a joint US-Polish study, spleen cells producing antibodies in mice administered a propolis extract were three times greater than controls. A second dose administered 24 hours later produced an even larger effect, although further doses reduced the effect [65].

Propolis was shown to increase antibody formation between two and three times that of controls in pigs vaccinated with "BUK-628" live Aujeszky's disease vaccine with and without addition of propolis. Antibody formation reached its maximum in 14 days, and antibodies could be detected for up to 330 days. Propolis also enhanced production of plasmacytes in the lymphoid tissue of the spleen and lymph nodes.

Propolis has been shown to suppress HIV-1 replication and modulate in vitro immune responses and, according to the authors, "May constitute a non-toxic natural product with both anti-HIV-1 and immunoregulatory effects" [66]. In mice, a concentrated extract of propolis has been shown to reduce blood pressure, produce a sedative effect, and maintain serum glucose [67]. Dihydroflavonoids as contained in propolis, have been shown to strengthen capillaries and produce antihyperlipidemic activity.

A strong immune deficiency was found in two patients with alveolitis fibroticans. Treatment with a

combination of the propolis, Esberitox N and calcium-magnesium resulted in good improvements in the state of the immune system and the clinical condition of both patients [68].

17.4.12 Antiviral Properties

A clinical trial has shown a prophylactic effect against influenza infection in humans [69], and another clinical trial showed that infections of the common cold were shorter in duration and completely recovered within 3 days in patients treated with propolis, compared to 5 days for recovery for patients not given propolis [70]. A clinical trial conducted on dermatology patients showed that a propolis cream had significant therapeutic effects against recurrent herpes (Herpes simplex type 1) and Herpes zona zoster (shingles). The propolis cream reduced duration of lesions and pain and increased interval between lesion episodes.

Perhaps the most broadly investigated and widely accepted attribute of bee propolis is its immune-boosting activity. It is a natural, broad-spectrum antibiotic that activates the thymus gland. Bee propolis not only prevents infectious diseases but clears them from the system as well. As demonstrated in numerous experiments, propolis has the ability to directly destroy bacteria, viruses, and fungi, even penicillin-resistant *Staphylococcus*.

17.4.13 Dental Care Properties

In rats inoculated with *sobrinus*, about half of fissures were carious, while dental caries were significantly less in rats given water containing propolis extract. No toxic effects of propolis on the growth of rats were observed under experimental conditions in this study [17]. Propolis has also been shown to be effective as a subsidiary treatment for gingivitis (gum infections) and plaque [71]. A 50% propolis extract was found to be antiseptic against pulp gangrene [72]. Propolis has also been shown to inhibit the growth of a range of bacterial organisms found in dental caries [73].

The diverse use of propolis in clinical trials showed that its therapeutic efficiency lies mainly in diseases caused by microbial contamination [74]. The results suggest that a propolis preparation can be a useful subsidiary treatment in oral hygiene.

A double-blind clinical trial showed that a propolis mouthwash (10% tincture diluted 1:5 with water) produced significant improvements in patients with gingivitis and periodontal disease. Patients were evaluated for plaque formation and inflammation of the gums. A clinical study used propolis extract and zinc oxide on 160 teeth with indirect capping or deep cavities and teeth with direct capping. The results showed that the

paste with propolis exerted effects similar to those of zinc eugenate and were superior for healing compared to pastes based on calcium hydroxide.

A clinical study found propolis useful for the treatment of gum inflammation and oral mucosa and also showed antiscarring effects [75]. Another study showed similar results for periodontitis and suggested that propolis be used in root canal fillings because of its bone regeneration and anesthetic properties.

17.4.14 Respiratory and Ear Infections

A total of 260 steel workers suffering from bronchitis were treated for 24 days by various methods including local and systemic regulation of the immune system and local treatment with an ethanol extract of propolis (EEP) in a physiological salt solution [67]. Promising results were obtained with inhalation of the extract, together with propolis tablets. Propolis has also shown positive effects in other otorhinolaryngologic diseases, such as pharyngitis chronic bronchitis [67], rhinopharyngolaryngitis [76], pharyngolaryngitis [77], catarrh [78], and rhinitis.

Patients (126) suffering from external otitis, chronic mesotympanic otitis, and tympan perforation were treated with propolis solutions (5–10%). A positive therapeutic result was reported in most cases. Propolis has also shown positive results in the treatment of acute inflammations of the ear [79].

17.4.15 Effects on Mitosis

Medical researchers (N. Popovici and N. Oita of Rumania) published a report on the effects of bee propolis on mitosis (the process of cell division). They reported that a tissue never becomes entirely malignant; it always contains some normal cells, but the activity of the normal cells is affected and even repressed by malignant cells. Propolis favors the activity of normal cells by repressing malignant cells, which helps the tissue to reestablish its normal condition. Constituents of propolis have a mitodepressive effect (depression of the proliferation of cancerous cells) on cells deranged by malignancy.

17.5 ROLE IN CELLULAR SIGNAL TRANSDUCTION

Signal transduction at the cellular level refers to the movement of signals from outside to inside the cell. The movement of signals can be simple, like that associated with receptor molecules of the acetylcholine class, receptors that constitute channels that, upon ligand interaction, allow signals to be passed in the form of small ion movement either into or out of the cell. These ion movements result in changes in the electrical potential

of the cells that, in turn, propagate the signal along the cell. More complex signal transduction involves the coupling of ligand-receptor interactions to many intracellular events. These events include phosphorylations by tyrosine kinases and/or serine/threonine kinases. Protein phosphorylations change enzyme activities and protein conformations. The eventual outcome is an alteration in cellular activity and changes in the program of genes expressed within the responding cells.

17.5.1 Classifications of Signal Transducing Receptors

Signal transducing receptors are of three general classes:

1. Receptors that penetrate the plasma membrane and have intrinsic enzymatic activity. Receptors that have intrinsic enzymatic activities include tyrosine kinases (e.g., PDGF, insulin, EGF and FGF receptors), tyrosine phosphatases (e.g., CD45 [cluster determinant-45] protein of T cells and macrophages), guanylate cyclases (e.g., natriuretic peptide receptors), and serine/threonine kinases (e.g., activin and TGF- β receptors). Receptors with intrinsic tyrosine kinase activity are capable of autophosphorylation as well as phosphorylation of other substrates. Additionally, several families of receptors lack intrinsic enzyme activity, yet are coupled to intracellular tyrosine kinases by direct protein-protein interactions.
2. Receptors that are coupled, inside the cell, to GTP-binding and hydrolyzing proteins (termed G proteins). Receptors of the class that interact with G proteins all have a structure that is characterized by seven transmembrane spanning domains. These receptors are termed serpentine receptors. Examples of this class are the adrenergic receptors, odorant receptors, and certain hormone receptors (e.g., glucagon, angiotensin, vasopressin and bradykinin).
3. Receptors that are found intracellularly and upon ligand binding migrate to the nucleus, where the ligand-receptor complex directly affects gene transcription. Because this class of receptors are intracellular and function in the nucleus as transcription factors, they are commonly referred to as the nuclear receptors. Receptors of this class include the large family of steroid and thyroid hormone receptors. Receptors in this class have a ligand-binding domain, a DNA-binding domain, and a transcriptional activator domain.

17.5.2 Signaling Molecules

Most signal transduction involves the binding of extracellular signaling molecules (and ligands) to cell surface

receptors. While triggering events inside the cell, such receptors typically face outward from the plasma membrane. Intracellular signaling cascades can also be triggered through cell-substratum interactions. One example is integrins, which bind ligands found within the extracellular matrix. Steroids are another example of extracellular signaling molecules that may cross the plasma membrane because of their lipophilic or hydrophobic nature [80]. Many, but not all, steroid hormones have receptors within the cytoplasm, and usually act by stimulating the binding of their receptors to the promoter region of steroid-responsive genes [81]. Within multicellular organisms, numerous small molecules and polypeptides serve to coordinate a cell's individual biological activity within the context of the organism as a whole. These molecules have been functionally classified as:

- Hormones (e.g., melatonin) [82]
- Growth factors (e.g., epidermal growth factor) [83]
- Extracellular matrix components (e.g., fibronectin) [84]
- Cytokines (e.g., interferon γ) [85]
- Chemokines (e.g., RANTES) [86]
- Neurotransmitters (e.g., acetylcholine) [87]
- Neurotrophins (e.g., nerve growth factor) [88]
- Active oxygen species and other electronically-activated compounds (see redox signaling)

Most of these classifications do not take into account the molecular nature of each class member. For example, as a class neurotransmitters consist of neuropeptides such as endorphins [89] and small molecules such as serotonin [90] and dopamine [91]. Hormones, another generic class of molecules capable of initiating signal transduction, include insulin (a polypeptide) [92], testosterone (a steroid) [93], and epinephrine (an amino acid derivative, in essence a small organic molecule).

The classification of a molecule into one class or another is not exact. For example, epinephrine and norepinephrine, secreted by the central nervous system, act as neurotransmitters. However, when secreted by the adrenal medulla, epinephrine acts as a hormone.

17.5.2.1 Artepillin C Derived from Propolis Acts as a Neurotrophic-Like Factor. It was investigated whether artepillin C, a major component of Brazilian propolis, acts as a neurotrophic-like factor in rat PC12m3 cells, in which nerve growth factor (NGF)-induced neurite outgrowth is impaired. When cultures of PC12m3 cells were treated with artepillin C at a concentration of 20 μ M, the frequency of neurite outgrowth induced by artepillin C was approximately sevenfold greater than that induced

by NGF alone. Artepillin C-induced neurite outgrowth of PC12m3 cells was inhibited by the ERK inhibitor U0126 and by the p38 MAPK inhibitor SB203580. It was proposed that artepillin C-induced activation of p38 MAPK through the ERK signaling pathway is responsible for the neurite outgrowth of PC12m3 cells [94].

17.5.2.2 Brazilian Propolis Suppresses Angiogenesis by Inducing Apoptosis Angiogenesis is defined as the process in which a network of new blood vessels emerges from preexisting vessels. Angiogenesis has been shown to be essential for tumor growth and metastasis, which are two major factors that hinder cancer therapy [95]. Food factors, such as epigallocatechin gallate (EGCg), indole-3-carbinol, resveratrol, and quercetin, possessed antiangiogenic properties [96–99]. Such antiangiogenic food factors could be used to effectively prevent small cancers from progressing.

Investigation of the effects of many angiogenesis inhibitors has revealed that one of the major antiangiogenic mechanisms of these drugs is induction of apoptosis in endothelial cells [100]. Apoptosis is a genetically programmed form of cell death. Angiogenic stimuli such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are known to activate extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt, which transduce survival signals in endothelial cells and simultaneously prevent apoptosis by inactivating proapoptotic proteins [101–103]. On the other hand, apoptotic stimuli are known to activate a caspase cascade that ultimately leads to the oligonucleosomal fragmentation of DNA and the cleavage of proteins such as poly(ADP-ribose) polymerase (PARP) and lamin A/C [104].

It was reported that ethanol extract of Brazilian propolis (EEBP) suppresses tumor-induced angiogenesis in vivo and tube formation of endothelial cells in vitro [105]. It has also been demonstrated that propolis suppresses angiogenesis through induction of apoptosis in endothelial cells [106].

Propolis suppresses tumor-induced angiogenesis through tube formation inhibition and apoptosis induction in endothelial cells. The schematic diagram of angiogenesis suppression by EEBP is shown in Fig. 17.1. It was also shown that EEBP and U0126 similarly induced activation of caspase-3 and cleavage of PARP and lamin A/C, all of which are molecular markers of apoptosis. These results indicate that inhibition of survival signal ERK1/2, and subsequent induction of apoptosis, is a critical mechanism of angiogenesis suppression by EEBP.

It is known that propolis inhibits tube formation and induces apoptosis in endothelial cells [106]. It was further confirmed that ERK1/2 inactivation was largely

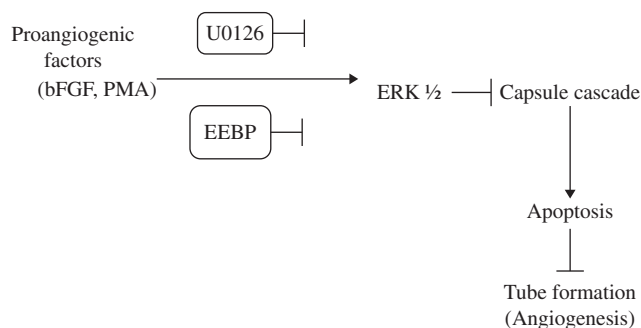


Fig. 17.1 Schematic diagram of angiogenesis suppression by EEBP, bFGF, and PMA (proangiogenic factors) stimulation of ERK1/2 signaling. The survival signal inactivates the caspase pathway, thereby maintaining cell survival facilitating angiogenesis.

responsible for antiangiogenic effects, tube formation inhibition and apoptosis induction, in endothelial cells.

ERK1/2 signaling in endothelial cells has been shown to play an essential role in angiogenesis both in vivo and in vitro. It was reported that bFGF and bone morphogenetic protein-4 induced the formation of capillary-like structures by endothelial cells through ERK1/2 activation [107, 108]. On the other hand, it was demonstrated that several pharmacological inhibitors, dominant negative constructs and siRNA against Raf/MEK/ERK pathway inhibited angiogenesis in vivo and tube formation of endothelial cells without affecting Akt activation [109, 110]. Such inhibitors have also been shown to induce endothelial cell apoptosis in vivo and in vitro [111, 112]. It was further confirmed that ERK1/2 inactivation alone is sufficient to prevent angiogenesis and induce apoptosis in endothelial cells. On this basis, it was suggested that ERK1/2 inactivation was a major mechanism responsible for antiangiogenic action of EEBP.

In this study, it was shown that EEBP inhibited ERK1/2 activation. It was reported that Brazilian propolis, collected from *Baccharis dracunculifolia* DC in Minas Gerais State, was composed mainly of artepillin C, caffeic acid, and *p*-coumaric acid [113]. Several constituents of Brazilian and Uruguayan propolis possessed antiangiogenic activities of varying degrees.

17.6 TOXIC EFFECTS

Propolis has been shown not to be toxic to humans or mammals unless very large quantities are administered [8]. Some of its constituent flavones, for example, quercetin, might be mutagenic by the Ames test, but mutagenicity per se for propolis has not been reported [6].

Contact dermatitis is a well-documented allergic reaction to propolis, with approximately 200 cases

reported in the literature over the last 70 years [114]. Initial reports were made by beekeepers, who came into daily contact with the raw product. Allergic reactions are now also reported in the general population, because of the more widespread use of products containing propolis. Dermatitis can be produced by skin contact with raw propolis as well as propolis extracts, and products containing caffeic acid and its derivatives have been identified as the major allergenic agent [115]. Cinnamic acid derivatives have also been implicated [65].

Dermatitis is relieved once the skin is no longer in contact with the propolis product. It is therefore recommended that with all preparations intended for human use, usage should be ceased whenever there is an allergic reaction. Very few other adverse reactions to propolis have been documented in the literature, and the product is generally considered not to be harmful [6]. Rare cases of oral inflammation and ulceration, mouth edema (swelling), and stomatitis have been reported as a result of oral ingestion of propolis [116, 117].

17.7 COMMERCIAL USE

Raw propolis is collected by beekeepers and sold in bulk to companies that refine the product and turn it into usable extracts. The main commercial uses of propolis are as a dietary supplement and therapeutic. Propolis is sold in tablets (singularly or in combination with other substances such as pollen, royal jelly and nonhive products). In Japan, the use of propolis is permitted as a preservative in frozen fish.

Tinctures and lozenges are popular treatment for sore throats, and tinctures are often used to treat cuts, mouth sores, and skin rashes. For internal use, a 1- to 3-ml dose three times daily of a 1:10 tincture is typical, but higher doses can be used if necessary. Propolis tincture is normally diluted in water, producing a cloudy liquid. For external use, the 1:10 tincture is diluted in water and used as a lotion.

Propolis is a stable product but should nevertheless be stored in airtight containers in the dark, preferably away from excessive and direct heat. Propolis does not lose much of its antibiotic activity, even when stored for 12 months or longer. Propolis and its extract function as a mild preservative because of their antioxidant and antimicrobial activities and thus may actually prolong the shelf life of some products.

17.8 FOOD SAFETY

Because of its antioxidant and antimicrobial activities, microbial contamination is not considered to be a

problem with propolis, either in the raw form or as extracts. Concentrations of lead above maximum allowable levels for food products have been found in propolis. Studies have shown that lead levels may be reduced by placing the hives away from areas with heavy air pollution and the use of oil-based paints on hive parts [118]. Propolis destined for commercial use should be routinely tested for lead concentration. Brazilian propolis is of the highest quality available, whereas Chinese propolis has been noted for excessive lead.

17.9 CONCLUSION

Propolis, bee glue gathered by bees from various plants, has been used by humans since early times for various purposes, and especially as a medicine because of its antimicrobial properties. At least 67 plant species have been reported to provide propolis material. Important sources include poplars, alders and birches, chestnut, ash, and willows. The class of compounds present in propolis includes resins, waxes and fatty acids, essential oils, pollen, and minerals. The resins comprise flavonoids, phenolic acids, and esters (45–55%), whereas essential oils contain 10% volatiles. There are nearly 12 free amino acids in pollen and 14 trace minerals, iron and zinc being the most common. The other organics present are ketones, lactones, quinines, steroids, benzoic acid, vitamins, and sugars. Propolis also has nutritive value because of the presence of small amounts of proteins, amino acids, minerals, and sugars. Vitamins include small amounts of A, B₁, B₆, C, and E. Because of its strong antimicrobial activity, propolis is often known as a “natural antibiotic.” Propolis is reported to possess anticancer, apoptotic, wound healing, antiinflammatory, antiulcer, antiviral, anesthetic, and immunomodulatory properties. Artepillin C, a major component of Brazilian propolis, was reported to act as a neurotropic-like factor and to suppress angiogenesis by inducing apoptosis. With regard to toxicity, propolis has been shown not to be toxic to humans or mammals unless very large quantities are administered.

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PART II

OXIDATIVE STRESS IN INVERTEBRATES

ENDOCRINE CONTROL OF OXIDATIVE STRESS IN INSECTS

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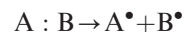
18.1 INTRODUCTION

The implications for the toxic properties of oxygen were unknown until Gershman's seminal proposal of the free radical theory of oxygen toxicity in 1954, which suggested that the poisonous nature of oxygen is due to the partially reduced forms of oxygen [1]. In the same year Commoner et al. [2] reported weak but detectable electron paramagnetic resonance (EPR) signal in lyophilized biological materials, and this was attributed to the presence of free radicals. These discoveries triggered intense research into the role of free radicals in biological systems. Since then, a large body of evidence has been accumulated that living systems have not only adapted to a coexistence with free radicals but have also developed various mechanisms for advantageous use of free radicals in various physiological functions. Oxygen free radicals generally termed as reactive oxygen species (ROS) as well as reactive nitrogen species are products of normal cellular metabolism. The harmful effect of ROS causing potential biological damage is called oxidative stress. This occurs in biological systems when there is an overproduction of ROS on one side and a deficiency of enzymatic and nonenzymatic antioxidants on the other. In other words, oxidative stress results from metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant and antioxidant reactions in living organisms.

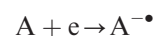
18.2 ENDOGENOUS SOURCES OF OXIDATIVE STRESS

Oxidative stress in insects is an unavoidable by-product of the aerobic lifestyle, because the superoxide anion ($O_2^{\bullet-}$) and H_2O_2 are formed whenever molecular oxygen chemically oxidizes electron carriers. The superoxide anion ($O_2^{\bullet-}$) radical is generated by the one-electron reduction of O_2 . Cellular sources of ROS production include plasma membrane NADPH oxidase and intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases, and mitochondrial electron transport components. ($O_2^{\bullet-}$) production can also be due to autooxidation of catecholamines, ubihydroquinone, hemoproteins, and flavin enzymes [3]. Other endogenous sources of ROS include stress and starvation. In all these cases the generation of free radicals is implied. A free radical is unstable and highly reactive and contains one or more unpaired electrons. It can be formed by any of the processes outlined below:

- a. Homolysis of covalent bonds



- b. Addition of a single electron to a neutral atom

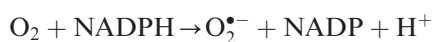
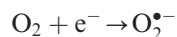


- c. Loss of a single electron from a neutral atom

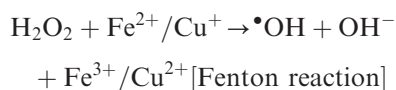
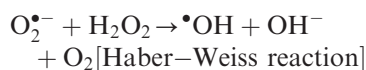


In insects the following free radicals are generated as a result of the above processes:

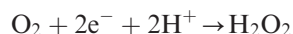
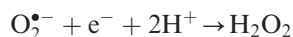
Superoxide anion generation:



Hydroxyl radical generation:



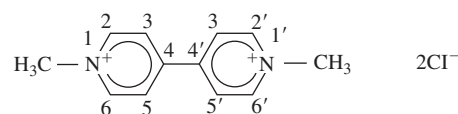
Hydrogen peroxide generation:



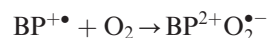
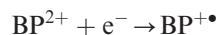
18.3 EXOGENOUS SOURCES OF OXIDATIVE STRESS

Environmental pollutants that include a variety of biochemical synthetic compounds used in industry and agriculture can affect nontarget organisms. These include metals, metalloids, and numerous other organic compounds. In general, halogenated alkanes and alkenes that are effluents from industrial sources and agriculture have been shown to induce oxidative stress by generating ROS in nontarget species. A comprehensive review of oxidative stress from environmental pollutants has been provided by Ahmad [4]. Here, we shall focus on oxidative stress in herbivorous insects that can be caused both by prooxidant plant allelochemicals and by various herbicides and/or insecticides. Plant phenolic compounds, particularly flavanoids and tannins, have long been associated with plant defense against herbivores [5]. Toxic phenoxyl radicals are formed via oxidative processes owing to their ability to initiate free radical chain reactions in the membrane and the propensity to cross-link with a variety of molecules [6, 7]. The midgut of insect herbivores is a highly oxidizing environment. Hence diet supplementation of lepidopteran larvae *Helicoverpa zea* and *Spodoptera littoralis* with phenolic acids was found to increase various indicators of oxidative stress in gut

tissues [8, 9]. The toxicity of phenolics results from several different modes of action including binding and oxygen radical formation. In general, phenolics can participate in four major types of bonds: hydrophobic, hydrogen, ionic, and covalent [10]. The formation of oxygen radicals is another important mode of phenolic action. Generally, almost any oxidation of phenolics can result in the generation of superoxide anion radicals because the reactive semiquinone can donate an electron to molecular oxygen. The superoxide anion so generated can further lead to the generation of additional radical species, including hydroxyl radicals, as described above. Thus, the propensity of phenolics to generate radicals depends on whether they are ionized or oxidized. The oxidation and ionization of phenolics depends on their phenolic structure, the physicochemical conditions under which the reactions take place, including hydrogen ion availability (pH), electron availability (E_h , or redox potential), and the concentration of antioxidant enzymes as well as nonenzymatic oxidants and reductants. Additionally, furanocoumarins are also known to generate free radicals in herbivores because they are photoactive prooxidants. These are found in plants of Apiaceae and Rutaceae. Among herbicides, paraquat is well known to generate oxidative stress in insect species [4].



Paraquat dichloride (BP^{2+})



18.4 DEFENSES AGAINST OXIDATIVE STRESS

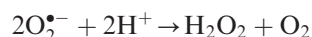
Defenses elaborated against oxidative stress are termed antioxidative defenses. By definition, then, an antioxidant can be defined as any substance that can either delay or prevent the oxidation of a substrate when it is present in small amounts relative to the amount of the substrate. According to Halliwell and Gutteridge [11], antioxidants can act at several different levels in the oxidative sequence, and they may have multiple mechanisms of action. Thus, antioxidative defenses could essentially be divided broadly into two main mechanisms: the enzymatic—which maintain harmless levels of activated oxygen species by reducing excess to H_2O —and the nonenzymatic—which are essentially free radical

scavengers designed to remove free radicals generated during reductive processes or during deleterious reactions between excess ROS and cellular macromolecules.

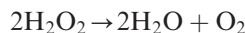
18.4.1 Enzymatic Antioxidative Mechanisms

Insects, like other animals, possess a suite of enzymes that are directed toward the removal of various radicals [12–17]. These include superoxide dismutase, catalase, ascorbate peroxidase, glutathione *S*-transferase peroxidase, etc.

Superoxide dismutase (SOD): Superoxide dismutases are mainly of two main types—the Cu/Zn SOD (located in the cytosol) and the MnSOD (localized in the mitochondria). These enzymes catalyze the following reaction:



Catalase (CAT): Catalase is a 24-kDa homotetrameric enzyme with a heme-iron active center, whose main function is to decompose toxic hydrogen peroxide [18]. This enzyme is localized notably in organelles such as peroxisomes. Hydrogen peroxide is eliminated by catalase as follows:



Ascorbate peroxidase (APOX): This enzyme catalyzes the oxidation of ascorbic acid with the concurrent reduction of hydrogen peroxide and could serve an important function in removal of hydrogen peroxide in insects [19]. Regeneration of reduced ascorbate is achieved by the enzyme dehydroascorbate reductase. Ascorbate by itself is known to scavenge singlet oxygen, superoxide, and hypochlorite as well as water-soluble radicals such as peroxyl, hydroxyl, thiyl, suphenyl, and nitroxide radicals [13,20–25]. Thus, in addition to its peroxidase activity, the efficient recycling of ascorbate itself gives it a powerful antioxidant role.

Glutathione S-transferase peroxidase (GSTPx): Vertebrates contain a selenium-dependent glutathione peroxidase (GPOX) that removes both hydrogen peroxide and lipid peroxides [12]. Insects have a glutathione *S*-transferase with peroxidase activity (GST-px). This enzyme can metabolize lipid peroxides but is unreactive toward hydrogen peroxide [12, 15, 26, 27].

Thioredoxin/thioreductase system (Trx/TrxR): To maintain its preferred redox state, the insect cells activate the NADPH-dependent thioredoxin/thioreductase enzyme system, which rapidly restores the modified thiols to their unmodified reduced state [25, 28].

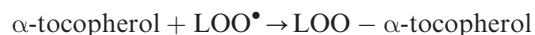
18.4.2 Nonenzymatic Antioxidative Mechanisms

In addition to the classical antioxidant enzyme systems, a number of small molecules also play a significant role

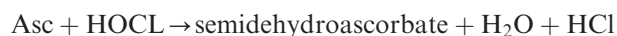
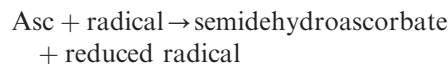
in scavenging ROS and curbing the deleterious effects of oxidative stress. Specifically in insects some of these small molecules are plant derived, while others can be synthesized by insects:

Carotenoids: The carotenoids constitute a family of conjugated polyenes. Carotenoids could be vitamin A precursors and are able to quench singlet oxygen. They may also directly react with peroxyl radicals. In plants their role has been well documented as a free radical scavenger, but the specific role in insect systems remains a speculation.

α-Tocopherol: The natural α-tocopherol is the 2R,4'R,8'R-α-tocopherol. This lipid-soluble vitamin is very reactive in membranes and can react with lipid peroxyl radical LOO• to form the relatively stable radical that probably does not react.



Ascorbic acid: Insects cannot synthesize ascorbic acid and depend on dietary sources for their supply of this essential antioxidant. In the presence of reactive species, ascorbic acid (or its conjugated base, ascorbate) is a one-electron donor and is oxidized in semidehydroascorbate radical, a molecule stabilized by the delocalization of the electrons between the three oxygen atoms. The semidehydroascorbate is converted back into ascorbic acid in the presence of glutathione and dehydroascorbate reductase:



Glutathione (GSH): Glutathione is a very important free radical scavenger. This is a tripeptide γ-glutamyl-cysteine-glycine and is one of the most abundant low-molecular-weight thiols present in the cell. Reduced GSH is characterized by its reactive thiol group, and as an effective reductant it plays an important role in a variety of detoxification processes. GSH readily interacts with free radicals and oxidizing compounds such as H₂O₂, O₂^{•-}, OH•, and carbon radicals including protection against lipid peroxide damage [14, 25, 29]. In the presence of the radicals the reduced form (GSH) is oxidized (GSSG) and can then be recycled back in a NADPH-dependent reaction catalyzed by glutathione reductase or by the thioredoxin reductase systems [30].

In addition to the antioxidant mechanisms and systems described above, insects also possess several water-soluble molecules (uric acid, carbohydrates, polyols) and iron binding proteins (ferritin and transferrin) that also serve crucial antioxidant functions [14].

18.5 REGULATION OF DEFENSES AGAINST OXIDATIVE STRESS

Oxidative stress triggers a range of physiological, pathological, and adaptive responses in insect cells either as a result of cellular damage or through specific signaling molecules. These responses ultimately modulate transcriptional outputs to influence and induce antioxidant systems. In the past couple of decades, a number of transcription factors and signaling pathways have been identified and delineated to mediate critical transcriptional responses to oxidative stress. These signaling pathways include mitogen-activated protein kinases (MAPKs) P13K/Akt, protein kinase C (PKC), protein53 (p53), nuclear factor κ B (NF- κ B), activator protein-1 (AP-1), and redox regulation by redox factor-1 (Ref-1) and the Nrf2-mediated antioxidant response. Nrf2 (nuclear factor erythroid 2 related factor 2) belongs to a group of specialized transcription factors termed xenobiotic-activated receptors (XARs). These recognize specific xenobiotics and coordinate the transcription of batteries of genes. The specifics of activation at the gene level of these signaling pathways are beyond the scope of this particular chapter. Here, we focus on the role that insect hormones play in the regulation of defenses against oxidative stress.

18.6 INSECT HORMONES AND THEIR ROLE IN THE CONTROL OF OXIDATIVE STRESS

The insect endocrine system produces various hormones and biologically active factors that can be divided into three main groups [31] including (a) ecdysteroids, steroid hormones, produced primarily by prothoracic glands and partially also by several other tissues. They control mainly molting, development, metamorphosis, and reproduction and are also involved in a number of diverse processes. (b) Juvenile hormones (JHs), a family of acyclic sesquiterpenoids, the principal products of the corpora allata—a retrocerebral gland—are involved in the regulation of metamorphosis and reproductive processes such as control of gonadal development and vitellogenin synthesis. Additionally, the roles of JH have expanded to include caste determination, behavior, diapause, and various polyphenisms [32]. And finally, insects also produce (c) neurohormones, a large group of peptidergic compounds produced by specialized secretory neurons, which are most abundant in the brain but do occur throughout the whole nervous system. They control a number of biochemical, physiological, and behavioral events in the insect body including those mentioned above for ecdysteroids and JHs [33]. The classification of neurohormones is not uniform, but they are ordinarily

categorized according to their functions. One of the best-defined groups of neurohormones associated with stress responses are metabolic neuropeptides belonging to the AKH/RPCH family (adipokinetic hormone/red pigment concentrating hormone family). A major function of these small peptides (octa-, nona-, or decapeptides), which are synthesized and released from an endocrine retrocerebral gland corpora cardiaca, is the control of insect metabolism. However, they are pleiotropic, with a number of actions in addition to their metabolic role. Generally, they behave as typical stress hormones by stimulating catabolic reactions (mobilize lipids, carbohydrates, and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. They mobilize entire energy reserves to combat the immediate stress problems and suppress processes that are momentarily less important and could, if allowed to continue, even draw on the mobilized energy. These biochemical stress reactions are accompanied by activation of physiological stress response that includes stimulation of heart beat [34], increase of muscle tonus [35], stimulation of general locomotion [36], enhancement of immune response [37, 38], and some others. Recently it has been found that AKHs are also involved in the control of oxidative stress (OS) in insects [see 39].

18.7 ROLE OF ADIPOKINETIC HORMONES IN INSECT OXIDATIVE STRESS

An active role of AKHs in protection of insects against OS was deduced from results of a set of papers published recently (see below). These results reveal that oxidative stressors increase the level of AKHs and, additionally, that exogenous AKHs mitigate OS biomarkers in insect body experimentally enhanced by application of the stressors. These facts indicate that there is a feedback regulation between an oxidative stressor and AKH actions, and that AKHs are involved in the activation of an antioxidant protection mechanism in insects.

Application of the oxidative stressors elevates the titer of AKHs in hemolymph. The effect is reported for paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride hydrate) [40, 41] a redox cycling herbicide that is commonly used to create conditions of OS in insect body, and also for *Galanthus nivalis* agglutinin and *Bacillus thuringiensis* toxin [40] and for endosulfan and malathion [42]—insecticides with an OS effect. The intensity of AKH level elevation varies depending on insect species, type of oxidative stressor, or time of the stressor incidence. For example, feeding of genetically modified potatoes containing the Cry 3Aa *Bacillus thuringiensis* toxin elevates the AKH level in hemolymph of the Colorado potato beetles *Leptinotarsa decemlineata* up

to 6-fold and treatment by paraquat about 2.7-fold within 4 hours after the treatment [40]. Similarly in the bug *Pyrrhocoris apterus* paraquat increases the AKH level in hemolymph about 5 times (again 4 hours after the treatment) [41] and insecticides endosulfan and malathion about 1.5 times 24 hours after the treatment [42].

Even larger variability was recorded when level of AKH was measured in the corpora cardiaca or the whole CNS (including the corpora cardiaca). In *L. decemlineata* feeding of both the Cry 3Aa toxin and *Galanthus nivalis* agglutinin elevates the level up to 10 times and application of paraquat just 1.9 times [40]. In *P. apterus* the same application has no effect on the AKH level in the CNS (4 hours after the treatment) [41], and treatment with the insecticides endosulfan and malathion elicits only a slight increase in the CNS [42]. On the other hand, the differences between the stress-evoked AKH levels in CNS and hemolymph are not so surprising, because the whole amount of AKH seems to be substantially distinct in those two body parts. It is reported there is about 200 times lower AKH amount in hemolymph than in CNS of *P. apterus* [43]. In addition, no positive correlation is shown for the same species between the AKH level in hemolymph and CNS [44]. This result is in good agreement with the suggestion that a coupling between release and biosynthesis of the AKHs in adipokinetic cells of the corpora cardiaca is very loose or does not even exist [45].

Questions arise on the possible mode of the feedback action on the biochemical pathways by which the enhanced AKH level mitigates the OS. While the mechanisms of AKH action leading to energy mobilization have been documented by a number of publications [46–48, etc.], almost nothing is known about the stress-evoked AKH actions that do not include rapid production and subsequent consumption of energy. Other than OS, stress-induced elevation of AKH titer was demonstrated in the locust *Schistocerca gregaria* and *P. apterus* challenged with an insecticide [42, 49–51], excessive KCl [49], and photophase interruption and exposure to constant darkness [52].

The suggestion that AKHs are involved in the activation of protective antioxidative mechanisms derived from the effect of oxidative stressors on AKH level in the insect body is supported by a series of experiments demonstrating direct involvement of AKHs in the modulation of OS biomarker levels. Only a few biomarkers have been studied in the relationship to AKH actions so far. One of the most convenient is glutathione. It is a low-molecular-weight thiol (see Section 18.4.2) found in the cytosol and other aqueous phases of various living systems [53, 54]. The level of GSH in insect hemolymph is significantly increased (about twice) after AKH injection but significantly depressed (about 2–3 times) after

paraquat treatment both in *L. decemlineata* [40] and *P. apterus* [41] hemolymph. On the other hand, coinjection of AKH together with paraquat results in enhanced GSH content back to the control level. A very similar picture is obtained when the insecticides endosulfan and malathion are used [42]. It appears that AKH is able to directly or indirectly enhance efflux of reduced GSH into the insect hemolymph as demonstrated for the pancreatic vertebrate hormone glucagon that stimulates massive GSH efflux from the liver into the bloodstream [55]. However, it is quite possible that GSH alone (induced by AKH) could not be the one to confer enhanced antioxidant capacity to the hemolymph.

Another important marker of OS is protein carbonylation, whose level illustrates the oxidative damage of the system. Carbonyls are formed from amino groups in the side chain of certain amino acids that are exposed to ROS [56]. Application of paraquat into both *L. decemlineata* and *P. apterus* body, as well as application of Cry 3Aa and *Galanthus nivalis* toxins in the food of *L. decemlineata*, significantly enhance carbonyl contents in hemolymph, but coinjection of paraquat with AKH decreases their levels to those found in control groups [40, 41]. It is interesting that AKH injection alone does not change the carbonyl contents to those below control values. This indicates that possibly a stressor action is needed for AKH to potentiate the response, as in case of phenoloxidase activity [37], lipid store mobilization rate after injection and/or topical application of external AKH [57], and modulation of catalase activity [42] (see also below). It seems that the adipokinetic response is enhanced and or to some extent modified primarily in the presence of stress, caused by an injection or by an application of insecticides as in the two latter cases.

A lowering of the carbonyl contents also supports the suggestion of an operative role of AKHs in antioxidant action to counter OS in insects. The complex strategy of this action is still unclear; however, it is quite possible that more players, besides GSH, are involved. This suggestion is supported by measurement of the total antioxidant activity in cell-free plasma of *P. apterus* against Trolox (an analog of vitamin E) standards. This procedure relies on antioxidant activity of low-molecular-weight compounds. The results show that paraquat injection alone potentiates an antioxidant response that is significantly enhanced upon coinjection of paraquat with AKH. However, AKH injection alone is not capable of inducing antioxidant activity to the levels produced by paraquat alone or by coinjection with paraquat, although it is more enhanced compared to control groups [41]. Hence, this supports the suggestion that there must be some other additional mechanism(s) by which AKH acts to enhance antioxidant response.

The list of AKH activities involved in the OS control also includes the modulation of activity of catalase that is responsible for decomposition of toxic hydrogen peroxide into water and oxygen [18]. Application of insecticides endosulfan and malathion causes a significant increase in catalase activity in the whole body of *P. apterus* but when coapplied with AKH a significant decrease is recorded compared with insecticides alone. The catalase activity after the coapplication is still significantly higher than that after AKH treatment alone; however, the fall as compared to application of the insecticides is substantial, achieving 2.3- to 3.7-fold lower values. Since free radical-scavenging enzyme complexes like superoxide dismutase, catalase, and glutathione peroxidase are in the first line of cellular defense against oxidative injury [58–60], the induction of catalase after insecticide application is not surprising. Possibly, AKH reduces the production of hydrogen peroxide by an unknown mechanism, and therefore the activity of catalase necessary for reduction of hydrogen peroxide is lower.

AKH activities in the stress elicited by insecticides are also linked to one interesting phenomenon: an enhancement of the insecticide activity by AKH coapplication. This phenomenon is probably not directly connected with OS, because it was first reported for the insecticide permethrin [51], which does not possess the ability to generate OS, but a certain relationship to OS cannot be completely excluded when endosulfan or malathion, known to promote formation of free radicals, is used [61–63]. The coapplication of the insecticides with AKH increases *P. apterus* mortality up to 3-fold [42]. Almost nothing is known about the mechanism of the phenomenon, but it has been suggested that the AKH stimulation of metabolism [33] could enhance the insecticide action. This suggestion is supported by the AKH-induced significant increase of carbon dioxide production [42,51]—an indicator of metabolic rate in the experimental bugs. The increased metabolic rate could intensify the insecticide action by faster penetration of the insecticide into tissues and by a more intensive exchange of metabolites affecting the biochemical pathways, including also the OS reactions. However, in the absence of direct data this statement remains a speculation.

18.8 ROLE OF OTHER INSECT HORMONES IN OXIDATIVE STRESS

Not only have AKHs been implicated to be involved in hormonal control of antioxidative protective reactions in insects, but other hormones such as glucagon, ecdysteroids and JHs have also been suggested to be involved.

Glucagon: It is a 29-amino acid peptide well-known as a vertebrate hyperglycemic hormone. In insects,

immunochemically similar glucagon-like peptides were reported in hemolymph [64], midgut [65, 66], and nervous system [66–69] of several insect species. On the other hand, a role for the glucagon-like peptides in insect body has not been explained satisfactorily. They could play a role in the brain-gut axis [70] and participate also in the control of digestive or other metabolic processes, but direct evidence is still missing. There are some indications that glucagon-like peptides play a role in regulation of insect glycemia, as was shown in the honey bee *Apis mellifera* [71], but this effect is most likely not common in other insects [72]. Analogous to vertebrates [55,73], Alquicer et al. [66] suggested for insects that glucagon could play a role in defense against OS, because injection of porcine glucagon into the *P. apterus* body elicits the antioxidant response by significantly increasing GSH and decreasing protein carbonyl levels in hemolymph, and decreasing both protein carbonyl and protein nitrotyrosine levels in CNS. Moreover, similarly as reported for the AKHs (see Section 18.7 for details), when coinjected with paraquat, glucagon partially eliminates OS markers elicited by this oxidative stressor and returns them to the control levels. Nevertheless, the lack of significant changes of AKH titer in *P. apterus* body after the injection of glucagon suggests that glucagon action is AKH independent.

Ecdysteroids: These are well-known insect steroid hormones playing a crucial role in insect developmental and reproductive events (see also Section 18.6). In vertebrates steroid hormones, namely, estrogens and related compounds possessing a phenolic A-ring, were shown to be involved in the control of OS. They inhibit the oxidation of cholesterol and the peroxidation of polyunsaturated fatty acids (diene conjugation or malondialdehyde formation) in the lipoproteins, microsomes, and other components of biological systems [74]. 20-Hydroxyecdysone, one of the most important member of insect steroid hormones, shows similar features. It has been proven that 20-hydroxyecdysone is a potent antioxidant able to minimize the OS impact of paraquat to *P. apterus* [17]. This ecdysteroid restrains lipid peroxidation and the formation of protein carbonyls, ameliorates changes in microsomal membrane fluidity, enhances the level of reduced glutathione, and upregulates the activity of γ -glutamyl transpeptidase in the brain. However, the protective effect of 20-hydroxyecdysone against the OS has also organismal dimensions. Certain hemolymph proteins, very sensitive to the paraquat treatment, are consistently present in the bugs being injected with 20-hydroxyecdysone despite the paraquat presence. The same injection ameliorates the suppressive paraquat effect on female fertility and in both sexes improves the survival rate curtailed by paraquat.

A similar function of 20-hydroxyecdysone is reported by Roesijadi et al. [75], who show that ecdysone induced methionine sulfoxide reductase A in the fruit fly *Drosophila melanogaster* is associated with enhanced resistance to hydrogen peroxide. Methionine residues in proteins are susceptible to oxidation when subjected to reactive oxygen or nitrogen species [76]. The reduction of the oxidized methionine form is catalyzed by methionine sulfoxide reductase [77]. Expression of the enzyme is regulated via the ecdysone receptor (EcR-UPS) complex [78] controlled by ecdysone. Overexpression of the enzyme is associated with enhanced protection against OS, while its knockdown results in hypersensitivity to OS [79, 80].

The mechanisms of action of these effects are unknown, but their existence indicates the importance of ecdysteroids for the management of OS in the insect body [17].

Juvenile hormones: These are insect terpenoid hormones with two main functions in insect life. As developmental hormones, they prevent premature initiation of insect metamorphosis in juvenile stadia and support reproduction by hormonally controlled gene expression of vitellogenins in adult females. Their role in OS seems to be indirectly mediated through the regulation of biologically active proteins like vitellogenins [81] and/or transferrin [82, 83].

Vitellogenins primarily serve as energetic and building components for a developing embryo in the insect egg. They are synthesized in specialized cells in the fat body or rarely in ovarioles by a complicated hormonally controlled process (see 84) in which the JHs in most insect species play a crucial role. In the bee worker vitellogenins protect the organism against the oxidative damage of paraquat [81]. The results of the authors show that vitellogenins are a preferred target of oxidative carbonylation in comparison with other hemolymph proteins, which is a property that is indicative of antioxidant function [85]. There is a direct implication of this finding for bee aging: Treatment of low- and high-vitellogenin level bee phenotypes with paraquat showed that survival was significantly lower for the former group. The data show that vitellogenin activity is causally linked to OS resistance of the bee, and further, they can explain why vitellogenins are synthesized at high levels in honeybee queens and are abundant in long-lived workers [81].

Insect transferrin is known as an iron transporter, regulated by vitellogenin and JH activities. Its essential function is transport of iron that is required for a wide variety of metabolic processes including oxygen transport and electron transfer; iron also participates in the Fenton reaction (see Section 18. 2), in which the very reactive hydroxyl radical is generated [86]. Insect

transferrin is regulated by a lot of diverse processes (87) including hormonal processes. As transferrin is suggested to be a vitellogenic protein, an involvement of the JHs is not surprising. Jamroz et al. [82] showed a strong suppression of the transferrin gene in the fat body by JH treatment of the cockroach *Blaberus discoidalis*. Harizanova et al. [83] demonstrated in the mosquito *Aedes aegypti* that the JH analog methoprene suppresses the increase of transferrin message after blood feeding. The reasons for the suppression are not properly understood: Transferrin gene could be a target of transcription factors of ecdysteroid-induced mosquito homologues, and a negative effect of JH on them could play some role [83].

18.9 CONCLUSION

The past couple of decades have yielded a plethora of literature advancing our understanding of the signal transduction pathways in response to oxidative stress. While these are important in mediating transcriptional response to oxidants at the molecular level, major players in stress response pathways such as stress-responsive hormones are only beginning to be implicated especially in insects. With this has also come an appreciation for the complexity of the endocrine-mediated responses and the awareness that these hormones may not be acting in isolation but intersecting with one another to mediate a myriad of the physiological and adaptive responses to oxidative stress in dose- and cellular context-dependent manners. Emerging from the survey of endocrine actions in stress responses (particularly oxidative stress) is a picture of extraordinary diversity and complexity, whether viewed in terms of the target cells, the metabolic pathways, or the physiological functions that the neurohormones regulate. Although we have tried not to be too elaborate, within our limited goal, we have discussed to the best of our available knowledge the role of insect stress hormones in oxidative stress. Molecular understanding of the interactions between the endocrine system and oxidative stress will continue to yield important insights into mechanisms that trigger such responses. We hope that the present review will stimulate further research within a framework of insect hormone actions to oxidative stress constituting a coherent, albeit complex and heterogeneous, physiological whole.

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OXIDATIVE STRESS IN THE AIRWAY SYSTEM OF THE FRUIT FLY *DROSOPHILA MELANOGASTER*

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19.1 INTRODUCTION

Oxidative stress is a major threat for all organisms living in a high-oxygen atmosphere. Reactive oxygen species (ROS) can be produced either exogenously or endogenously. The major exogenous source of ROS is the atmosphere, which implies that especially those organs or parts of the body that come into close contact with large amounts of air are at risk. This would mean that the airway epithelium is prone to be damaged by ROS and thus needs specialized mechanisms to cope with this situation. Endogenous production of ROS is highest in those tissues with the greatest energy consumption, as it is an inevitable by-product of mitochondrial activity. To cope with this ROS confrontation, animals employ different sets of enzymatic as well as nonenzymatic antioxidants. ROS-mediated damage of tissues and cells is believed to be one major reason for changes associated with aging. The oxidative stress theory of aging that was introduced more than 50 years ago [1] predicts that effective scavenging of ROS may delay aging and thus should increase life span. Free radicals and other ROS induce molecular damage at different levels, leading to increased incidences of mortality as a function of time. These ROS-mediated damages that can occur in all major groups of macromolecules accumulate over time, and, at a certain time-point, the organism is unable to repair all of them. Accumulation of these damages interferes with the normal physiological function of the cells, which may end up in reduced overall performance

of the organism and finally in mortality. A great number of studies have supported this theory, especially those performed with model organisms such as the fruit fly *Drosophila melanogaster*. In general, increasing the antioxidant repertoire of these model organisms tends to increase life span. In particular, two enzymatic antioxidants, superoxide dismutase (SOD) and catalase, tend to mediate life-prolonging effects in *Drosophila* or *Caenorhabditis elegans*, an observation that is still matter of debate.

19.2 OXIDATIVE STRESS SYSTEMS IN *DROSOPHILA*

A number of different tissues show a higher sensitivity toward oxidative stress than others. Among these are the nervous system, but also the airway epithelium, as this structure is constantly exposed to high oxygen flow rates and therefore to ROS. Regarding nonenzymatic antioxidants that are employed by the fly, our knowledge is limited. Interestingly, chronic food supplementation with compounds having antioxidant properties has the potential to increase life span, indicating that nonenzymatic antioxidants are of relevance for various aspects of the fly's biology [2]. In addition to these nonenzymatic antioxidants, a very comprehensive armamentarium of enzymatic antioxidants can scavenge these compounds, thus protecting the organism. Among these compounds are superoxide dismutases, catalase, peroxiredoxins,

thioredoxins, and the plethora of enzymes involved in glutathione metabolism. These enzymes are either constitutively present in different organs of the fly or the expression of the corresponding genes is induced after exposure to various stressors.

A large number of signaling pathways are involved in the response to oxidative stress and are therefore necessary to mount an adequate antioxidative response. In most of these cases, these cellular responses comprise not only oxidative stress resistance but also a general stress resistance. Among these signaling pathways that are of central importance for a proper response to oxidative stress is the PI3-kinase pathway, which converges onto activation of the transcription factor FoxO [3]. FoxO activation leads to expression of for example, 4E-BP, which is required for a proper and efficient expression of other target genes, mainly those associated with an increase in oxidative stress resistance. An alternative way of signaling is represented by the Nrf2/Keap1-pathway (Fig. 19.1). This is a system that appears to be tailored to sense oxidative stress and to induce an adequate cellular reaction. In unstressed conditions, Nrf2 is kept in the cytoplasm by its inhibitor Keap1. Keap1 has also the potential to act as a sensor for oxidants, by reacting with these compounds via redox-sensitive cysteine residues [4].

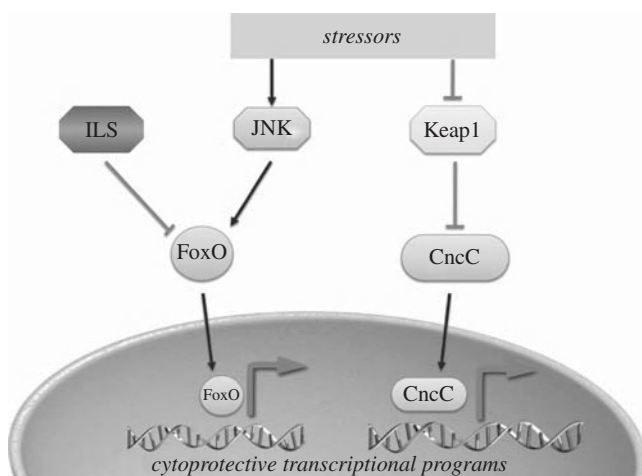


Fig. 19.1 The two transcription factors FoxO and Nrf2 (CncC) are activated by stressors and induce a cytoprotective transcriptional program operative in the airway epithelial cell. FoxO can be activated by a variety of stressors via the JNK pathway, whereas it is inhibited by the insulin-like signaling pathway (ILS). After activation, FoxO translocates into the nucleus, where it initiates transcription of various genes, e.g., those coding for enzymatic antioxidants. On the other hand, Nrf2 (CncC in *Drosophila*) is usually inhibited by Keap1 and held back in the cytoplasm. After stress application, Keap1 is inhibited, thus releasing CncC and allowing it to translocate into the nucleus, where it also starts a cytoprotective transcriptional program (e.g., transcription of glutathione-*S*-transferases).

Thus oxidants lead to dissociation of the complex between Nrf2 (CncC in *Drosophila*) and Keap1, allowing Nrf2 to translocate into the nucleus, where it binds to the antioxidant response element (ARE), an enhancer sequence present in the target genes of Nrf2 activation. Expression of these target genes represents the so-called electrophile counterattack comprising a whole battery of enzymatic antioxidants of all flavors. Among these are thioredoxins, enzymes of the glutathione metabolism, but also chaperones and parts of the proteasome [5]. In the fly, the Nrf2/Keap1 system fulfils all the tasks described above, and persistent activation of Nrf2 signaling via depletion of Keap1 increases resistance against oxidative stress and life span.

Other signaling systems known to be relevant for the resistance against stressors in general are also known to have an impact on the resistance against oxidative stress. The *Drosophila* p38 ortholog, which is an essential part of the p38 mitogen-activated protein kinase (MAPK) cascade, is essential for a reaction and the resistance against oxidative stress, but also for the resistances against heat stress and starvation [6]. p38, as one of the major parts of the MAPK pathway, has a high potential as a target for intervention in chronic inflammatory diseases including rheumatoid arthritis or asthma, but the side effects caused by chronic interference with its normal function are still not well understood.

19.3 REACTIVE OXYGEN SPECIES AND AGING

A direct connection between aging and oxidative stress induced damages was the idea behind one of the first theories of aging [1]. Accumulation of these damages over time leads to impairment of cellular function and finally to death of the organism. Studies from different fields show a connection between aging and ROS-mediated impairments of various different features. Aging and the reaction to oxidative stress share a number of identical transcript signatures. In particular, genes coding for enzymatic antioxidants and immune relevant genes are upregulated under these two different conditions [7]. Nevertheless, it must be kept in mind that the correlation between oxidative stress-mediated impairments and the physiological decline of various abilities during aging is not necessarily direct [8]. Decline in sensory perception occurs at the same rate in control flies and those showing a high oxidative stress resistance. Although the contribution of single parts of the antioxidant armamentarium to life span is hard to quantify, it is common sense that a highly effective antioxidant response is beneficial. A relatively large number of enzymatic antioxidants appear to have a positive effect on longevity if present in the organism. Among these

are, for example, the thioredoxins; corresponding knockout flies lacking a functional Thioredoxin-2 have a shortened life span [9]. A comparable effect on life span has been attributed to a peroxiredoxin of type II, named Jafrac1, whose overexpression in the nervous system appears to increase life span whereas its downregulation has the opposite effect [10]. Interestingly, Jafrac1 is a target gene of the JNK pathway that is activated after stress and cellular damage to restore the cellular homeostasis. Other peroxiredoxins, especially those present in mitochondria, are also relevant for life span, as their downregulation has a significant impact on this aspect of life [11].

Regarding the role of enzymes involved in glutathione metabolism, the picture is relatively complex because of the large number of gene products being part of this machinery. Glutathione reductase increases resistance under hyperoxic conditions, whereas no effects on life span could be observed at normoxia [12]. The hypothesis that glutathione metabolism is involved in life span regulation was further supported by the observation that overexpression of the glutamate-cysteine ligase, which is the rate-limiting enzyme in glutathione de novo synthesis, increases life span by more than 20% [13].

Two other sets of enzymatic antioxidants have been implicated in life span extension, the SODs and catalase. In particular, the role of SODs in this context is still a matter of debate. Overexpression of a human SOD1 in motoneurons only was shown to be sufficient for a 40% life span extension [14]. As mentioned above, overexpression of SODs was shown to increase life span significantly, but this effect appeared to be restricted to short-living strains of *Drosophila melanogaster* [15]. A combined overexpression of SOD and catalase appears to represent a more robust way of life span extension compared with overexpression of either of the two components [15, 16]. This observation is in line with the enzymatic activities of both enzymes that act in common.

Hyperoxia is a relatively simple way to induce oxidative stress. Although very high oxygen concentrations are lethal to naive flies, it was possible to select for survivors under these hostile conditions. These flies show phenotypical differences compared with control flies, and their gene expression profile was altered accordingly. Very interestingly, genes belonging to the family of antimicrobial peptide genes are obviously relevant for this adaptation to extreme oxygen concentrations and therewith to higher levels of oxidative stress [17, 18].

The plethora of signaling and effector molecules involved in the response to oxidative stress indicates that this response is of vital importance for the organism. Apparently, this response is tightly controlled and obviously of vital importance, especially regarding life span-associated aspects.

19.4 OXIDATIVE STRESS IN THE NERVOUS SYSTEM

Most important among these is obviously the nervous system, where ROS-mediated decline of neuron survival can be observed. As could have been expected, one class of neurons shows highest sensitivity to oxidative stress, namely, the dopamine-producing cells. Obviously, the dopamine production machinery is inevitably linked to an endogenous production of relatively high amounts of ROS, thus making these cells prone to damages caused by “extra doses” of ROS. This high sensitivity is the major reason why dopamine-producing neurons die whereas other neurons are almost unaltered [19]. This highly interesting observation enables us to link high ROS concentration with the development of Parkinson disease-like phenotypes. One major reason for the development of this disease is believed to be chronic confrontation with very high ROS concentration in conjunction with the sensitivity of dopamine-producing cells in general [19]. Consequently, confrontation with ROS doses is a suitable mechanism to induce neurodegenerative processes as typically seen in Parkinson disease. One of the most reproducible ways to do this is hyperoxia, which consequently is able to induce these phenotypes [20]. Especially based on these approaches, it was possible to verify hypotheses that ROS-scavenging enzymes should reduce the sensitivity to these high ROS levels in corresponding *Drosophila* models. SOD overexpression was sufficient to protect the dopaminergic cells, whereas catalase was not [20]. In addition, increasing the glutathione-S-transferase activity in the nervous system protects these dopaminergic neurons in another *Drosophila* model of Parkinson disease [21].

In an Alzheimer disease model based on tau-activation in the nervous system, ROS have been shown to modulate the sensitivity of the animals, thus demonstrating that oxidative stress plays a major role in the development of Alzheimer disease [22]. The positive effects of enzymatic and nonenzymatic antioxidants on disease progression appear not to hold true for all neurodegenerative diseases. In a well-established model for Huntington disease, neither overexpression of enzymatic antioxidants nor supplementation with nonenzymatic antioxidants decreased the lethality in this model [23].

19.5 OXIDATIVE STRESS IN THE DIGESTIVE SYSTEM

The intestinal immune system is characterized by a very special situation. It faces one of the most dense

populations on earth, the intestinal microbiome. Thus the major regulatory challenge for this organ is to hold a homeostatic balance between fight against potential pathogens and maintenance of the microbial community within the intestinal tract. Intestinal immune reactions are therefore different from those of other epithelia such as the airway epithelium. Whereas the latter reacts quickly and strongly with the expression of antimicrobial compounds to confrontation with bacteria, the intestinal epithelium is refractory to this type of stimuli and reacts only relatively weakly. To complement this type of response and to fight potential pathogens, the intestinal immune system acts in a completely different way. Fighting pathogens is achieved via enzymes producing ROS. Most important among them is obviously the dual oxidase (DUOX) that is secreted from enterocytes in response to pathogen contact [24, 25]. DUOX is a member of the nicotinamide adenine dinucleotide oxidase (NADPH oxidase) family. Surprisingly, the intracellular signaling pathway transducing the recognition of pathogens into the secretion of DUOX has nothing to do with classical immune relevant pathways such as the Toll or the IMD pathway. It comprises signaling pathways well known from neurotransmitter or hormonal signaling systems, namely, G-protein coupled receptor systems involved with the G α_q G-protein in a central position [26]. Downstream is the phospholipase C- β , also known as norpA. Animals defective in PLC- β have a shortened life span due to massive proliferation of an otherwise harmless microbe, the yeast *Saccharomyces cerevisiae*. This yeast is the major nutritional microbe in most *Drosophila* diets and has usually no pathogenic potential. The observation that PLC- β mutants, which are consequently unable to release DUOX, lost the ability to control this bug indicates that DUOX plays a very important role in maintenance and shaping of the microbiome [26]. The receptor transmitting these effects is yet not known, but activation of this pathway leads to IP $_3$ production and subsequent Ca $^{2+}$ release from internal stores, which in turn is the trigger for DUOX release into the intestinal lumen. DUOX-defective mutants have a phenotype very similar to that of PLC- β -defective mutants. They are hypersensitive to proliferation of bacteria, even of nutritional bacteria that are otherwise harmless. Yet-unknown bacterial products, also acting through the unidentified G protein-coupled receptors mentioned above, trigger regulation of DUOX expression and release into the gut. The killing mechanism of DUOX comprises the production of H $_2$ O $_2$, which is a typical feature of DUOX enzymes in general [27]. Although ROS production by DUOX is an important way to fight pathogens, some of these are resistant to oxidative stress; for this type of pathogens, NF- κ B-mediated

expression of antimicrobial peptide genes is a complementary type of reaction.

Oxidative stress produced by the epithelial cell is well suited to kill most of the invading bacteria but has the disadvantage that the organism's own tissue can be heavily injured. Thus the fly must have a two-sided type of response: While export of ROS-producing enzymes is important; protecting the fly's own epithelium from ROS-mediated damage is also. To ensure this type of concerted action, an enzymatic antioxidant, immune-regulated catalase (IRC), is produced to protect the cells from ROS-mediated damage. Consequently, IRC is required to survive contact with bacteria in the intestine, even if they are heat killed. These bacteria induce a massive ROS reaction that, if IRC is not there, can cause fatal damage to the cells of the intestine [25]. In addition, other mechanisms operative in the intestinal epithelium protect the organism's own cells from ROS present in the intestinal lumen. Sensing of this activates the JNK pathway, which in turn leads to activation of cellular adaptations allowing the organism to cope with this situation. ROS confrontation induces a JNK-triggered autophagy reaction of the enterocytes that helps to keep them alive under these otherwise very hostile conditions [28].

19.6 OXIDATIVE STRESS IN THE IMMUNE SYSTEM

Production of highly effective ROS is a versatile method to fight pathogens that is used by a great variety of cells of the innate immune system. The oxidative burst produced by, for example, macrophages is the most impressive example highlighting this strategy. Thus this way to combat pathogens is primarily used by motile cells of the innate immune system. In insect immunity, especially in the fruit fly's immune system, this type of response, namely an oxidative burst, has not been shown to be operative. Only the hemocytes, the motile cells of the immune system that have the ability to phagocytose invaders, come into consideration for this task, but they appear not to employ this strategy.

Nevertheless, ROS play an important role in the differentiation of hemocytes. In *Drosophila*, multipotent hematopoietic progenitors, the stem cells of the hemocyte lineage, react to different ROS levels with a speeding up or arresting of their differentiation [29]. In these progenitor cells, ROS levels are slightly enhanced, presumably to sensitize them for further differentiation processes. Further increasing the levels of ROS leads to a precocious differentiation into the final cells of this lineage (all three different types of hemocytes). Involved in the transduction of ROS levels into developmental

signals are obviously both the JNK and the FoxO pathway. In particular, the role of FoxO is interesting, as it usually induces the expression of genes that produce oxidative scavengers such as enzymatic antioxidants.

It has been suggested that ROS defense and immune response do not always act in the same direction. One particular peroxiredoxin, namely, Prd5, appears to have the capacity to modulate the immune response. Interestingly, knockout flies are more resistant to infection, whereas flies overexpressing this gene are more susceptible. Interestingly, these effects seem to be mediated via the JNK pathway, which has the capacity to link “damage” signals with the immune response of the fly [30].

One special aspect of the role of oxidative stress in immunity can be attributed to phenol oxidases (POs). POs are enzymes that produce a melanin coat surrounding invaders of different natures. These enzymes are produced as proenzymes, and are thus called pro-phenol oxidases (PPOs). In *Drosophila* three different PPOs are present (PPO1–3), which are predominantly expressed in different hemocyte subtypes. During melanization, these enzymes are able to produce a locally high concentration of ROS, which may be one of the major effectors of the PPO system. The conversion from PPOs to PO that occurs via limited proteolysis is tightly controlled because the end product, melanin, is highly toxic. This conversion is triggered by the recognition of bacterial patterns including peptidoglycans. Surprisingly, only for PPO3, can the conversion into the enzymatically active form PO be achieved without additional stimuli. Overexpression of this form of PPO was sufficient to induce massive melanization in almost every tissue targeted [31]. PPO1 and 2 are primarily expressed in crystal cells that are constitutively present in the hemolymph and react strongly to injury. These cells can release PPO1 and 2 to induce melanization in the hemolymph. PPO3, in turn, is restricted to lamellocytes that are usually not found constitutively in the hemolymph but are induced in the presence of larger intruders, such as parasitoids that inject their eggs into the body cavity of larvae. These cells surround these intruders and form a melanizing barrier to immobilize and finally kill the intruder [32]. Taken together, it is not known to what extent ROS are involved in the PO-mediated killing of invading microorganisms or larger intruders, or whether ROS production is only an inevitable by-product of melanin synthesis.

19.7 OXIDATIVE STRESS IN THE AIRWAY SYSTEM

The airway system of the fly has a very peculiar structure, made of interconnected tubes in a hierarchical order. First-order tubes, second-order tubes, and final

branches make up the entire system. This organization guarantees that almost every cell of the body has direct access to an oxygen supply (Fig. 19.2). Throughout the entire system, a single layer of epithelial cells surrounds a central air-filled lumen. The corresponding cells are seemingly uniform in their response characteristics, while fulfilling the major tasks of airway epithelial cells, enabling an effective oxygen transport and exchange. These cells react to different stimuli with appropriate responses including massive responses if pathogens are experienced (Fig. 19.2). Oxygen is transported through this system to all parts of this body, causing a reasonable oxidative stress. In principal, all oxygen transport systems are prone to damage caused by high oxidative stress because these cells have direct contact with the air and large volumes of air pass over their surface during the process of gas exchange. Our knowledge regarding the effects on the airways and mechanisms of the airway epithelium to counteract this stressful situation is almost nonexistent.

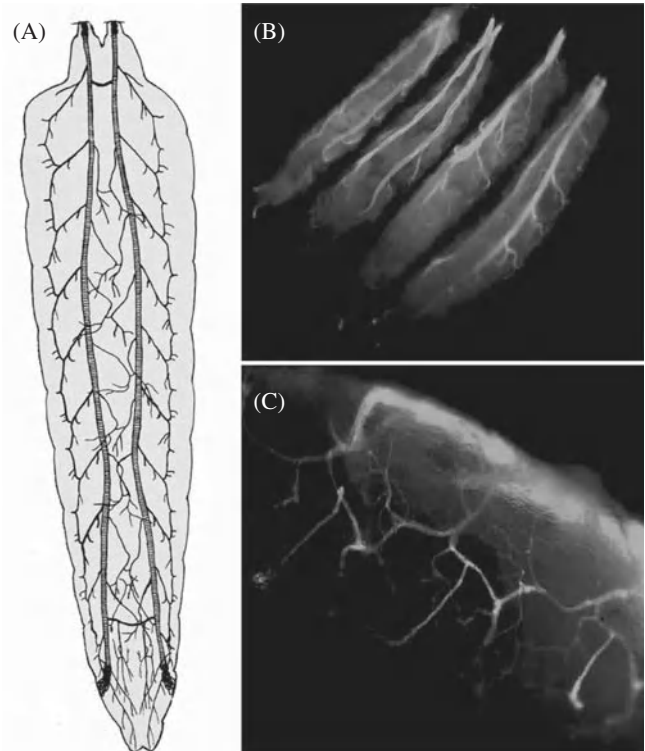


Fig. 19.2 Structure of the airway system of *Drosophila*. The airway system (trachea) of a larval fly is made up of interconnected tubes that deliver oxygen to almost every cell in the body (A). Upon stimulation with different stressors including infection, the airway epithelium launches a very effective response, comprising the expression of antimicrobial peptide genes (B and C, the latter at higher magnification). (See color insert.)

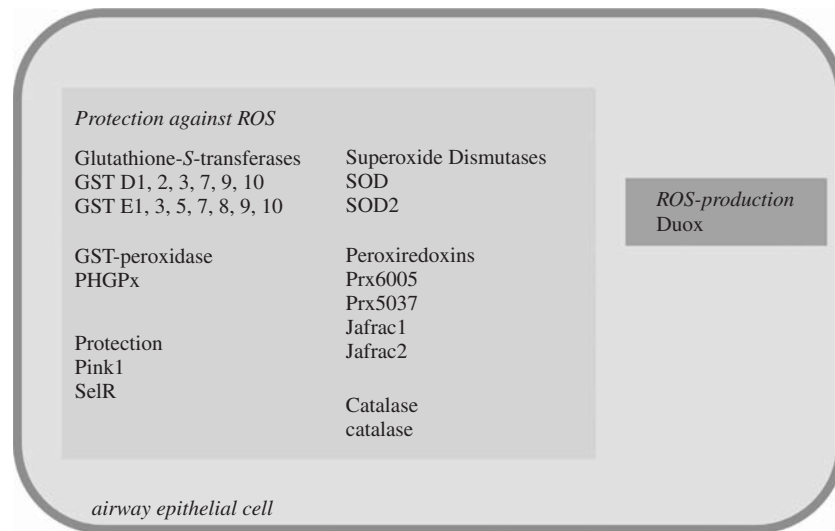


Fig. 19.3 Schematic drawing of reactive oxygen species (ROS)-producing enzymes and those protecting from ROS in the airway epithelial cells of the fly.

A recent study was performed with the aim of determining the relationship between oxygen concentration, oxidative stress, and life span [33]. Counterintuitively, the relation between these parameters is not linear, and reduced life spans were observed at maximal and minimal oxygen concentrations in the atmosphere.

It has been shown that all major enzymatic antioxidants are expressed in the airway epithelium. Among these, superoxide dismutase (SOD), catalase (cat), and all molecules involved in glutathione metabolism are of greatest importance. These enzymatic antioxidants are not only expressed in the airway epithelium but are also part of the reaction to stressful situations. The only well-studied situation is the reaction to airway infection [34]. In addition to classical aspects of an antibacterial response including of antimicrobial peptide genes, some of these enzymatic antioxidants are strongly upregulated.

The complex armamentarium of enzymatic antioxidants comprises both SODs (SOD and SOD2), the sole catalase, four different peroxiredoxins (Jafrac 1 and Jafrac 2, Prx 6005 and Prx 5037), a big consortium of glutathione-S-transferases (GST D1, 2, 3, 7, 9, 10, GST E1, 3, 5, 7, 8, 9, 10), the glutathione-peroxidase PHGPx, and molecules involved in protection such as *PINK1* and *SelR* (Fig. 19.3). A very potent producer of reactive oxygen species, the dual oxidase Duox, which is known to be very effective against invading bacteria, supplements this armamentarium. Thus the antioxidant system of the airway epithelium contains both producers of ROS and protectors against them. This architecture of oxidant and antioxidant systems allows the organism to cope with the very peculiar system in the airway, namely, that very high ROS concentrations have to be tolerated by these very peculiar cells.

19.8 CONCLUSION

ROS are an omnipresent threat for almost all cells in the body. In particular, those organs with highest metabolic rates and those directly exposed to the atmosphere are in danger. Thus the airway epithelium especially has to cope with this situation. To overcome problems associated with ROS-mediated damage, corresponding cytoprotective systems must be installed. In particular, enzymatic antioxidants including SOD, catalase, peroxiredoxins, thioredoxins, and those involved in glutathione metabolism are relevant. Although the vast majority of the corresponding genes are transcribed constitutively, different stressors can induce their expression further via the JNK-FoxO axis or the Nrf2 system. Our knowledge regarding their physiological role under different conditions is limited, although we know the almost complete armamentarium of antioxidants in the airway epithelium. With the experimental advantages of the *Drosophila* system, it should be possible to obtain an in-depth understanding of this very important regulatory system.

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MOLECULAR MECHANISMS OF ANTIOXIDANT PROTECTIVE PROCESSES IN HONEYBEE *APIS MELLIFERA*

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20.1 INTRODUCTION

Features of the honeybee evolutionary development have defined a special role of oxidative stress and the molecular mechanisms of its regulation in the processes associated with homeostasis preservation by an individual and a bee family.

Normally, reactive oxygen species (ROS) are produced continuously and moderately in cells. A cell neutralizes them with the help of the antioxidant system and replaces damaged molecules. A level of ROS exceeding the protective capabilities of a cell can cause a serious damage, even the cell's death. Thus oxidative stress as the cost of an aerobic life is a constant cause or an important component of many problems for the organism.

Nevertheless, oxidative stress is actively used by insect organism as a defense mechanism to neutralize xenobiotics and fight against pathogens in the processes of morphogenesis and life span regulation. Some ROS can act as messengers through redox signaling. In this situation, the system of antioxidant protection of a honeybee provides a wide range of vital important functions, including both protection against oxidative damage and adjusting an individual's life span in the colony. Moreover, eusociality obtained by individual phylogenetic groups of insects determines many peculiarities of the functional use of ROS and the molecular mechanisms of their regulation in the honeybee

organism, as well as significant restructuring in the functional orientation of genome expression.

This chapter considers the role of oxidative stress in the process of determining the preservation of homeostasis by both a honeybee organism and a colony.

20.2 ANTIOXIDANT SYSTEM OF HONEYBEE

20.2.1 Honeybee as Insect

In the process of aerobic organism evolution, complex antioxidant systems, including enzymatic and nonenzymatic components, have been formed to prevent oxidative damage. There exist primary and secondary antioxidant enzymes that affect ROS molecules directly or indirectly.

Defense against the destructive action of ROS is first provided by three groups of primary antioxidant enzymes by direct action. Superoxide dismutase (SOD) converts superoxide anion radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2), which is then neutralized by catalase. Peroxidases catalyze similar reactions in which H_2O_2 is reduced to H_2O with the use of reduced thioredoxin (TRX) or glutathione (GSH) as an electron donor.

Key components of the antioxidant defense system are stored in organisms of all evolutionary branches, but there are unique adaptations peculiar to individual groups [1]. In insects, as compared to vertebrates and

other phylogenetic groups, genes encoding glutathione reductase (GR) and glutathione peroxidase (GPX) are absent. Their functions are performed by homologous genes encoding thioredoxin reductase (TrxR) [2] and thioredoxin peroxidase (TPX) [3]. In addition, insects have genes that encode enzymes of antioxidant defense that act as peroxidases: phospholipid-hydroperoxide GPX homologs with TPX activity (GTPX) [4] and glutathione *S*-transferases (GST) [5, 6]. Thus the group of secondary antioxidant enzyme in insects acting on ROS indirectly includes TrxR, which converts both TRX and GSH, and also methionine sulfoxide reductases (MsrA and MsrB), which are involved in protein reparation by catalyzing the TRX-dependent conversion of methionine sulfoxide to methionine [7, 8].

Insects have an open circulatory system, so a free flow of ROS in the body can not only lead to foreign agent destruction but also can be very dangerous for the insect organism. Simultaneous functioning of the systems producing and neutralizing ROS allows on the one hand stopping the development of infection and on the other hand preventing the insect organism from total intoxication.

20.2.2 Honeybee as Species

Coding of the complete genomes of several species of insects allows us to make a preliminary comparison. The honeybee genome has been found to evolve more slowly than the genomes of the fruit fly and mosquito. In this case, the honeybee genome with respect to genes

involved in circadian rhythm, RNA interference, and DNA methylation has more similarities to the genomes of vertebrates than to the genomes of the fruit fly and mosquito. At the same time, compared to *Drosophila melanogaster* and *Anopheles gambiae*, there are fewer genes presented in the genome of *Apis mellifera* associated with innate immunity, detoxification proteins, and gustatory receptors but more genes associated with the odorous substance receptors [9–11]. There are absolutely unique genes responsible for collecting and processing nectar and pollen. New microRNAs have been discovered that are expressed depending on the stage of development and specialization of bees; this means that these microRNAs participate in the honeybee social diversification [12]. To date, 39 genes coding 10 groups of antioxidant proteins have been identified in the honeybee genome (Table 20.1).

Special attention is paid to vitellogenin, the glycolipoprotein egg yolk precursor, involved in the development of reproductive functions of an insect female and a nutrient of the bee brood, which performs an antioxidant function in the honeybee organism [23–26]. Antioxidant properties of vitellogenin are stipulated by its Zn-binding capacity [22] and preferential oxidative carbonylation under oxidative stress in bees [23]. With respect to these properties, vitellogenin is compared to Cu/ZnSOD, key metal binding antioxidant enzymes also undergoing preferential carbonylation [27], and serum albumin, metal binding proteins that can function as free radical acceptors and reduce levels of oxidative markers such as protein carbonylation [28].

TABLE 20.1 Antioxidant proteins of the honeybee

Antioxidant proteins	Antioxidant Functions	References
Superoxide dismutase: mitochondrial MnSOD cytoplasmic Cu/ZnSOD	Reduction of $O_2^{\bullet-}$ formed in mitochondria to O_2 and H_2O_2	1, 13, 14
Catalase: cytoplasmic extracytoplasmic (in honey)	Reduction of $O_2^{\bullet-}$ to O_2 and H_2O_2	1, 13, 15
TPX (peroxiredoxin)	Reduction of H_2O_2 with the use extracytoplasmic (in honey) of TRX as a donor of e^-	1, 3, 16, 17
GTPX	Reduction of H_2O_2 and organic hydroperoxides	1, 4
TrxR	Transfer of reducing equivalents from NADPH to TRX and GSH disulfide with formation of powerful intracellular antioxidants—thiol-based reductants	1, 2, 18
TRX	Preservation of redox homeostasis homologs of TRX of the cell	1, 2, 18
Glutaredoxin (GRX) homologs	Preservation of redox homeostasis of the cell	1, 2, 18
GST	Metabolism of xenobiotics and protection against peroxidative damage	1, 5, 6, 13, 15, 17, 19–21
MsrA and MsrB	TRX-dependent reduction of methionine sulfoxide to methionine, participation in protein repair	1, 7, 8
Vitellogenin	Predominant carbonylation	22, 23

20.3 ROS AS COSTS OF AN AEROBIC METABOLISM

The main sources of ROS in any organism are the mitochondria (respiratory chain), microsomal oxidation of xenobiotics, and phagocytosis.

20.3.1 Mitochondria

ROS as by-products of aerobic metabolism are being continuously formed in the cells of honeybees under normal physiological conditions, and they necessitate constant monitoring of the antioxidant system. Two main reactions take place with the formation of ROS in the mitochondrial respiratory chain: the formation of $O_2^{\bullet-}$ from O_2 and its dismutation (disproportionation) under the influence of MnSOD with the formation of H_2O_2 . In addition to the reactions occurring in the electron transport network of the inner mitochondrial membrane, the source of ROS is the oxidative deamination of biogenic amines with the formation of H_2O_2 under the influence of monoamine oxidases localized in the outer membrane of mitochondria. This reaction contributes significantly to the creation of a stable concentration gradient of ROS between the mitochondrial matrix and cytosol [29, 30]: The concentration of $O_2^{\bullet-}$ in the mitochondria in the norm is 5–10 times higher than in the cytosol and nucleus. Exceeding this level, and hyperproduction of H_2O_2 , being relatively a long-lived molecule that easily diffuses through the mitochondrial membranes, creates the preconditions for the oxidative damage to mitochondrial matrix. According to some researchers, the intensive formation of H_2O_2 in mitochondria leads to disruption of intermolecular interactions and damage of the mitochondria inner membrane, as has been shown in studies of mitochondria isolated from muscle tissue of synanthropic flies in model systems generating ROS [31, 32]. Mitochondrial DNA is the most vulnerable to the damaging effect of ROS because it is in close proximity to the ROS-generating sites and is not protected, like the nuclear DNA, by histone proteins. The extreme sensitivity of the mitochondrial DNA to the damaging effect of ROS may lead to an increase in the number of mutations and the consequent suppression of aerobic respiration, since mitochondrial DNA encodes carrier proteins of the electron transport network.

20.3.2 Microsomes

Another source of ROS in the honeybee organism is the microsomal oxidation of xenobiotics. Microsomes contain enzymes of the system of cytochrome *P450* (CYP), which catalyze polyvalent oxidation of xenobiotics with

simultaneous generation of $O_2^{\bullet-}$ and other ROS. Two phases are distinguished in the process of xenobiotic clearance: the introduction of polar groups with the help of the CYP hydroxylase system and the conjugation of molecules with water-soluble ligands. Both processes are used to eliminate foreign components from the internal environment of an organism. The CYP hydroxylase system includes flavoproteins and a family of hemoproteins, which are localized on the cytoplasmic side of membranes of the endoplasmic reticulum. Different isoforms of CYP are involved in metabolism of various xenobiotics [33, 34]. Two groups of CYP are distinguished: The first is involved in the metabolism of endogenous substances; the second is induced by exogenous agents. The conjugation processes are often catalyzed by UDP-glucosidase, sulfotransferase, and GST. Glucuronidation is the major form of conjugation for agent detoxification. Sulfation usually provides a lowering of toxicity and acceleration of xenobiotic clearance. The GST reaction is important for neutralization of unstable electrophilic molecules. Microsomal GST is closely linked with the CYP system, which contributes to rapid inactivation of active metabolites produced during the metabolism of xenobiotics.

20.3.3 GST of Honeybee

The genome of the honeybee, in contrast to other insects, contains significantly fewer genes of xenobiotic detoxification protein. This may be due to the evolution of the hormonal and chemosensory processes and is a genetic payment for the highly organized eusociality of the honeybee. The most notable features of the honeybee are manifested in the coding of three important families of xenobiotic detoxification enzymes: Honeybees have only about half the genes of GST, CYP, and carboxyl/cholinesterases as other insects [35–37]. In particular delta and epsilon GST and CYP4, which contribute significantly to the binding of insecticides in other species, are insufficient. This deficit can contribute not only to honeybees' increased sensitivity to insecticides but to insufficient resistance to oxidative stress caused by incomplete microsomal oxidation of xenobiotics [38]. Foragers suffer from it more, because other members of the colony are relatively isolated from external influence in the hive.

Other insect GSTs have been assumed to play a minor role in protection against insecticides, only attenuating the effects of oxidative stress. However, lately it has been shown that GST, belonging to the Delta and Epsilon classes, are directly involved in the disposal of insecticides. The complete absence of class Epsilon enzymes in the honeybee and the presence of only one enzyme of class Delta GSTD1 [19, 39] may partly explain the

extreme sensitivity of this species to certain insecticides. Perhaps GSTD1 can serve as a universal mechanism for the protection of bees against harmful xenobiotic action. This enzyme is also involved in protection against oxidative stress. It is available in the spermatheca of the queen bee, and it is assumed to protect the sperm from oxidants.

In contrast to the lack of genes Delta and Epsilon GST, the honeybee has more genes of class Sigma. These enzymes were initially assumed to play an important role in the structural organization of muscle tissue [20], but enzymes of class Sigma GST turned out to have a high affinity to the products of lipid peroxidation and are localized in metabolically active tissues, such as those in the thorax muscle of flies. They are hypothesized to play an important role in protecting these tissues against oxidative stress by-products [21]. Omega, Theta, and Zeta classes of GS, are presented in bees as well as in other organisms. Probably, they play a key role in metabolic processes, in contrast to a predominantly detoxifying role of the Delta and Epsilon GST. This hypothesis is supported by the participation of the first in the degradation of products of tyrosine and phenylalanine metabolism and the importance of Omega GST in removing of S-thiol adducts from proteins [36]. Despite the fact that microsomal GST and cytosolic enzymes may play a similar role in the overall reaction of detoxification and protection from oxidative stress, they do not have structural similarities and are different in genetic origin [40].

Increase of activity of microsomal mixed-function oxidases and GST is recorded as the initial response of foragers to the action of pesticides and allelochemicals contained in toxic nectar, and the increase of the detoxifying activity depends on the age of worker bees [41, 42].

Thus the features of the cytochrome oxidase spectrum in the honeybee are mostly connected with the limitation of their function in xenobiotics metabolism and a great accentuation on hormones metabolism. As noted above, this aspect has a particular evolutionary significance for the honeybee.

20.3.4 ROS as Component of Immunity

Protection of insects against pathogenic microorganisms is carried out by phagocytic cells of hemolymph. The unlocked type of circulatory system promotes development of rapid generalized response to any influence. Metabolic activity, the so-called respiratory burst of phagocytic cells, is a source of formation in the NADPH oxidase reaction of $O_2^{\bullet-}$, triggering a cascade mechanism of ROS generation (Fig. 20.1). The latter have expressed microbicidal activity and are part of the cytotoxic

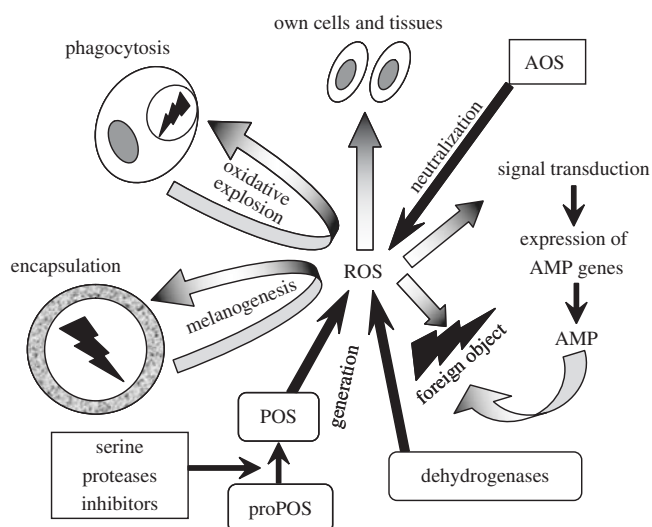


Fig. 20.1 Immune processes of the honeybee with ROS generation and neutralization. POS, phenoloxidase system; AOS, antioxidant system.

arsenal of the humoral immune system of honeybees [43, 44]. ROS are generated in the insect hemocytes with the participation of several enzyme systems—a phenoloxidase system (POS), a respiratory cascade, and a complex of dehydrogenases.

20.3.5 ROS and Phenoloxidase Cascade

Phenoloxidase (PO) is one of the oxidases responsible for the main part of the oxygen uptake during initiation of immune responses, as well as in morphogenetic processes of insects. The defeat of the honeybee by pathogens is accompanied by an increase of levels of proPO gene expression and PO activity [45–48]. In general, the power of the honeybee immune response and its ability to resist infection directly depend on the level of PO activity [49]. Quinone intermediates of melanin, $O_2^{\bullet-}$, H_2O_2 , hydroxyl radical ($\bullet OH$), nitric oxide ($\bullet NO$), and peroxynitrite ($ONOO^-$), involved in the cytotoxic reactions of insects in granule formation and encapsulation, are formed in large numbers during the realization of the PO cascade and melanogenesis in the hemolymph of insects [50–52].

From the immunological point of view it should be noted that biogenic amines (as mediators of stress reaction), as well as quinones, melanin, and other highly reactive metabolites formed during the activation of PO directly or indirectly participate in such defense reactions of insects as hemolymph coagulation and wound healing, encapsulation, granule formation, and destruction of the pathogen penetrated into the body by ROS. Components of the POS are also involved in the process

of immune recognition, carrying out cross-links of the foreign cell surface with the corresponding phagocyte receptors [53]. Uptake of foreign material by phagocytes is accompanied in both insects and vertebrates by an increase in oxygen consumption with active formation of ROS, which is analogous to the oxidative explosion [54, 55].

In addition, ROS generated during activation of PO and phagocytosis act as mediators of signal transduction, inducing gene expression of antimicrobial peptides (AMP) [51, 56, 57]. The presence of genes encoding proteins of the Toll-pathway of signal transduction in *A. mellifera* [58] suggests the existence of the same process in the honeybee.

However, if killer mechanisms of AMP are directed exclusively against bacterial and fungal cells, ROS have cytotoxic effects on all living cells, including cells of the insect organism itself. To reduce the cytotoxicity of ROS to the insect organism itself, its level is regulated by inhibitors of the proPO cascade, such as serine protease inhibitors [58] and components of the antioxidant system (AOS) [59, 60].

20.3.6 Interaction of Phenoloxidase and Antioxidant Systems

Correlation between functioning of the POS and antioxidant mechanisms in the defensive response of insects has been shown in a number of studies. The initial stage of infection in the honeybee has been established to be accompanied by activation of a PO cascade and inhibition of antioxidant enzymes activity [61]. The activity level of PO, catalase, and peroxidase in the honeybee imago changes under the action of the immunomodulator chitosan [62]. An analogous reaction of PO and AOS

has been found under bacteriosis in lepidopterous larvae [52] and adults of Colorado potato beetle [63].

In the above-mentioned papers it is assumed that the temporary inhibition of the organism's own AOS may play an important role in antimicrobial immunity of insects through the production of ROS generated in the implementation of the PO cascade (see Fig. 20.2). However, a different explanation of these changes in enzymatic activity can be supposed. The PO cascade may act mainly on the pathways of the formation of quinones, serving as a trap for oxygen radicals and reducing the concentration of ROS in hemolymph and, therefore, the activity of enzymes neutralizing them [64].

Study of the interaction between different antioxidant enzymes under the action of honeybee pathogens has revealed a similar pattern. A marked differentiation between catalase and peroxidase functions has been shown in the hemolymph and gut [65, 66]. Utilization of H_2O_2 at the early stage of infection development is carried out by catalase, whereas in the later stages of pathogenesis peroxidase gets involved. An inverse correlation is observed in the development of these reactions.

20.3.7 Social Immunity

The above defense responses are components of individual immunity of the honeybee and are encoded by genes, being only one-third of the number of immune genes of nonsocial insects [58]. This payment for eusociality is compensated by effective social immunity, including health behavior and the secretion of antiseptic substances in the larval food and honey, as well as in the internal environment of the hive.

Glucose oxidase, catalyzing the oxidation of β -D-glucose to D-glucose with the formation of H_2O_2 and

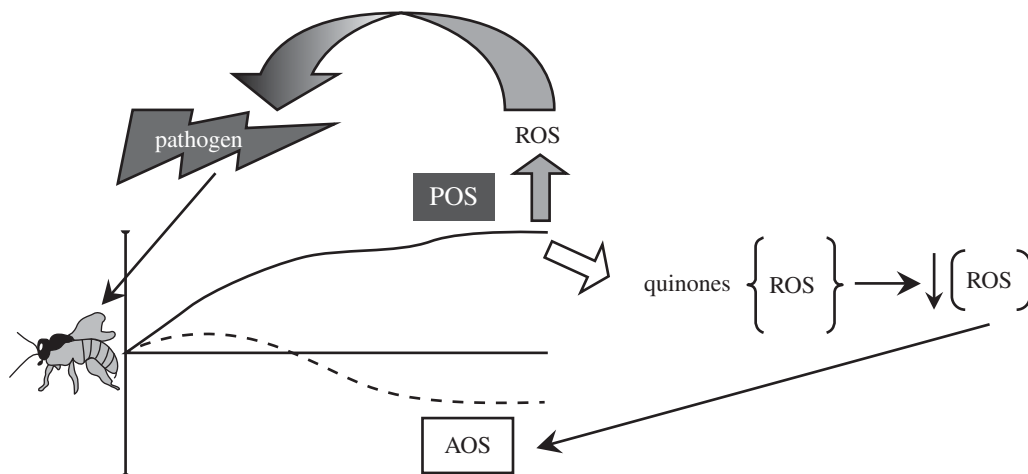


Fig. 20.2 Correlation of phenoloxidase and oxidative systems in honeybee defensive responses.

being a biochemical indicator of the level of social immunity of honeybees, plays an important role in providing of antiseptic properties of bee products [67]. Its activity is inhibited by the reaction product. The presence of this enzyme in honey with a large amount of substrate creates a relatively constant and self-regulating bactericidal system [68]. Importance of glucose oxidase in the group protection of honeybees is accentuated by the direct dependence of the colony strength on the level of this enzyme activity: H_2O_2 stipulates the antiseptic properties of the larval food and honey, inhibiting pathogen development [69, 70].

However, catalase activity, supporting H_2O_2 concentrations less toxic to the honeybee are found in honey. It is noteworthy that the genome of the honeybee contains only the gene of the intracellular catalase. The fact that the gene of catalase contained in honey is not integrated into the genome of the honeybee and extracellular catalase is found only in some bacteria and fungi suggests the expression of extracellular catalase of honey by endosymbiotic organisms [1].

Another component of the larval food—royal jelly—also contains highly active antioxidant substances that can reduce cadmium-induced genotoxicity and oxidative stress in mice significantly [71].

20.4 ONTOGENESIS AND LIFE SPAN

Oxidative stress and the molecular mechanisms of antioxidant protection of the honeybee motivate a particular research interest in connection with aging and longevity of animal organisms. The honeybee, because of the caste subdivision of the family, age-related functional specialization, and different life spans of the castes, is a natural model for gerontological research. According to the free radical theory of aging, the main reason for this process is the accumulation of ROS, oxidizing proteins [72], lipids, and DNA [73] and thus leading to DNA damage, degradation of membrane proteins, disruption of signaling pathways, and ultimately necrotic or apoptotic cell death [74].

Ontogenesis of the honeybee, which is a holometabolous insect, is characterized by a change of stages that are significantly different in morphology, physiology, and functioning. The transition from one stage to another is provided by major morphogenetic transformations occurring during metamorphosis and accompanied by the development of cellular and humoral reactions characteristic of anticontagious response [75]. Change in hormonal balance during periods of metamorphosis of the larvae and pupae, the appearance of the macrophagocytes in the hemolymph which are involved in the processes of histolysis, is also

accompanied by the analog of the oxidative explosion and the induction of molecular mechanisms to restrain it. Changes in the activity of SOD, catalase, and GST in the development of different insects implies that the cellular antioxidants are involved in protecting cells against damage and regulation of redox levels in the process of insect morphogenesis and are hormone dependent [76–78]. In honeybees during the first 3 days of embryonic development the level of thioredoxin peroxidase and GST expression significantly increases, which indirectly confirms the assumption of an active generation of ROS due to the high oxygen demand in the developing embryo [17]. Catalase activity reaches its highest values in the intestine of fertilized queens, which is associated with their more active feeding and significantly greater life span [13].

Ontogenetic changes in the activity of immune factors of the honeybee are under hormonal control [22, 25, 79] and can be related to the varying ability of the honeybee for antiinfective protection at different stages of development. Evolutionarily established social relationships in a colony of honeybees exhibit age-related features of cellular protection in individuals. In the honeybee the total number of functionally active hemocytes increases at the larval and pupal stages of development [49], which confirms their active involvement in morphogenetic processes. During the maturation of adults immunocompetence of the hemocytes decreases regardless of bees' sexual and caste identity [80], and levels of POS and AOS activity increase [15, 49].

Relatively low resistance to pathogen action and short duration of life are beneficial in terms of viability of the bee colony. In accordance with the "law of 40 days," bees of the second summer generation (May–June) are grown in large quantities on a relatively poor diet for mass participation in the honey harvest. According to the Institute of Beekeeping in Russia, from June 20 to August 1 bees collect 90% of all feed stocks [81]. At the end of the main honey harvest the existence of worn-out "unemployed" foragers becomes unprofitable for the colony. Drone removal from the colony takes place at the same period.

In the period when worker bees start forage conservation, the intensive death of hemocytes is accompanied by increase of juvenile hormone titers and a simultaneous decrease in the vitellogenin level [23, 79] (see Fig. 20.3). It is remarkable that an active cell proliferation, accompanied by a fall in titer of endogenous juvenile hormone and increasing of vitellogenin levels, starts in the hemolymph of foragers that are forced to return to hive work [82].

In general, an increase in ROS generation and enhancement of antioxidant protection is associated with aging and transition of worker bees to foraging.

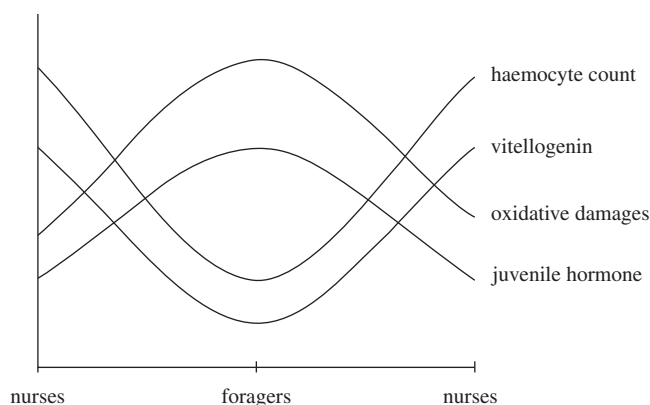


Fig. 20.3 Hormone-caused plasticity of worker bee defensive reactions.

Flight of foragers, as one of the most aerobically consumed types of activity, causes production of a large amount of ROS in the thorax muscle cells [83, 84], and a significant increase of Cu/ZnSOD content to neutralize $O_2^{\bullet-}$ [14]. In the midgut of worker bees the activity of catalase, GST, and microsomal oxidase of mixed function increases with aging [15, 41, 42], which reflects the conjugacy of detoxification and oxidative stress systems, being a result of foraging flight and associated contact with various pollutants.

The aging process is correlated with the accumulation of carbonylated proteins in the brain of foragers [23]. Carbonylation of proteins—the introduction of carbonyl groups in protein chains by direct oxidation or reaction with ROS—is one of the main oxidative modifications of proteins associated with biological aging [85].

Accumulation of carbonylated proteins is not the direct function of the chronological age of the worker bees, appearing in bees only after a long period of foraging activity [23]. Thus the processes of biological aging are associated not only with the real life expectancy of the honeybee, but with its behavior and functions performed in a colony.

Antioxidant enzymes also play an important role in the longevity of germinal cells. The sperm of drones is stored for a long period in spermatheca of a fertilized female, maintaining respiratory activity and thus running the risk of oxidative damage. However, sperm remains viable for many months, which is explained by a high activity of antioxidant enzymes in the reproductive tissues of the honeybee. Thus, in female spermatheca after fertilization, both the level of gene transcription of catalase and GST [19] and the activity of these enzymes greatly increase [13]. In addition, a high catalase activity in the spermatozooids and drone semen [13] and high levels of antioxidant-encoding transcripts in the reproductive organs of males [19] are associated.

20.4.1 Vitellogenin

The life span of the honeybee is closely associated with the functioning of vitellogenin, which has a positive effect on cellular immunity, preserving the integrity of proteins and increasing resistance of insects to oxidative stress [22, 23, 80]. A feature of long-lived bees is a high concentration of vitellogenin in the hemolymph. The highest level of vitellogenin is registered in the ovaries of queen bees, where it performs its main function in the development of oocytes [26]. Nevertheless, a high level of vitellogenin content in the tissues of queens is, apparently, one of the factors of their extreme longevity (several years) in comparison with other castes of the honeybee.

Vitellogenin is synthesized in hypopharyngeal glands of worker bees at the nurse stage and is present in the brood food [86]. The level of vitellogenin production in worker bees is much lower than that in queens [23]. However, the vitellogenin level could rise significantly as a result of its prolonged synthesis in worker bees with a low titer of juvenile hormone and insignificant use at brood withdrawal from the family [22]. Under natural conditions the honeybee subspecies *A. m. mellifera* and *A. m. carnica*, inhabiting the temperate zone, differ from subspecies of subtropical and tropical zones by the presence of temporary forms of worker bees, which accumulate vitellogenin to the levels in the queen and live for 6–10 months [79]. These facts suggest that the long-lived phenotype of the “winter bees,” which allows the bee family to survive in the winter period with no restoration of the strength at the expense of the brood, appeared through developing of the ability to accumulate vitellogenin.

Honeybee vitellogenin can affect cellular and biochemical processes by modulating the signaling pathways affecting the metabolism in general [23], and thus it influences the aging process [87]. It starts a series of cascade processes acting as a factor regulating the endocrine system of insects in general through stimulation of insulin-like peptide. Vitellogenin influences the ratio of titers of juvenile hormone and 20-hydroxyecdysone [88]. The changes also concern the level of expression of the transcription factor dFOXO in the fat body [89, 90], which has a significant impact on the reproductive system of females and their resistance to oxidative stress. A group of FOXO proteins plays an important role in response to various kinds of stress and regulates a wide range of cell reactions through a change of metabolism, from the retardation of cell cycle and differentiation processes to aging and apoptosis. It defines the role of FOXO-dependent mechanisms in determination of the honeybee's life expectancy. Activation of the insulin/IGF-1-pathway leads to stopping of FOXO activator function. Under stressor activity, the insulin pathway is

inactivated and JNK and SIRT1 is induced. Dephosphorylated transcription factor FOXO moves from the cytoplasm to the nucleus, which stops cell growth and, in turn, leads to increase of resistance to oxidative stress (FOXO-regulated genes include genes encoding SOD and catalase) and life expectancy [85, 88].

The molecular bases of complex social behavior of social insects are still vague. The gene encoding vitellogenin required for the formation of insect eggs performs a number of other functions in the honeybee that are related to social labor organization. Vitellogenin protein affects the age at which a worker bee stops working inside the colony and starts foraging, when it will collect nectar or pollen, as well as its duration of life [79]. It is possible that such behavior could occur because of desynchronization of the work of genes responsible for reproduction and care for the brood. Thus parental instincts of worker bees appear before their reproduction period starts. With no brood of their own, such insects have been forced to take care of closely related members of the colony [91].

Gene regulators initially associated with reproductive function, for example, influencing the production of gonadal hormones, can be expected to play an important role in the regulation of labor organization in the honeybee [92]. Vitellogenin gene in insects, both social and nonsocial, is closely linked to female reproductive function: Protein encoded by this gene is necessary for the formation of eggs in the ovaries of females. However, this gene is also active in worker bees that do not oviposit. Moreover, its activity in worker bees has been observed to decline with age. This means that vitellogenin serves as a singular behavioral “switch”: Decrease of its concentration forces a bee to leave household chores and to start foraging outside the hive at some moment of life. Thus the same gene that was originally associated with female reproductive function started to perform a number of new functions in the honeybee such as regulation of labor organization and social structure.

It has been found that in worker bees a wide range of enzymes involved in the processes of glycolysis, ATP synthesis, and generation of free radicals depends on the changes of tasks performed by the bees: from functions inside the hive to collecting of nectar or pollen [14, 93]. Evolution of the insect social castes with different reproductive potential is also associated with changes in metabolism that affect the life span [94]. From this point of view, evolutionary development of the honeybee supposes a positive connection of vitellogenesis and life span, which is not regulated only for the queen bee. This occurred because the fertility and longevity of the queen bee determines the viability and development of the colony [95]. Thereby, the honeybee is an example of how socialization in the process of evolution and changes

in the scheme of reproductive potential control in the colony may be interrelated with life prolongation [94, 96–98]. This assumption is based on analysis of the interdependence of vitellogenin levels and life expectancy of the queen bee, as well as the dynamics of reproductive protein and structure of longevity in worker bees.

In addition, use of vitellogenin for stress resistance management of worker bees has been evolutionary confirmed. In worker bees vitellogenin begins to synthesize when health functions in the nest are performed prior to the function of feeding [99]. However, the level of synthesis of vitellogenin in worker bees is lower in comparison with that of the queen [100]. The protein content is depleted in the period from the performance of health functions by the working bee to larvae feeding [24]. Thus the absence of brood is a necessary but not sufficient condition for the emergence of long-living worker bees [79, 82]. Modulation of regulatory mechanisms of vitellogenin on the reproduction and maintenance of homeostasis may be recombined in queens and worker bees during the migration of the species to temperate regions [101, 102]. This recombination of functions between longevity increase and stress resistance contributed to the emergence of the phenotype of long-living worker bees, which promoted the maximum survival of the colony during the winter, when the worker bees could not be replaced because of environmental restrictions on brood raising.

20.5 WINTER GENERATION OF HONEYBEE

Before human intervention the species *A. mellifera*, having mastered West Asia, Africa, and Europe, was scattered so widely that its further evolution was in radically different natural climatic conditions. This factor led to the formation of 25–30 subspecies [103], differing in a number of features, having a specific reaction to the external environment, and often not even able to exist under artificial latitudinal displacement within the species area [104].

It is known that honeybee defensive reactions are characterized by clear interspecific differences [65, 66]. Honeybee subspecies differ in expression of antimicrobial peptide genes, the dynamics of PO activity, and hemocyte response [67, 105–107]. Interspecific differences are manifested in the reactions of the defensive systems of the honeybee to bacterial contamination, expressed in increased phagocytic reaction and an earlier activation of redox processes in *A. m. mellifera* in comparison with *A. m. caucasica* [108]. These results confirm that during the honeybee's evolution modification of adaptive properties did not concern individuals, but a colony in general as an integral biological unit [91].

A phenomenon of the winter generation of bees, which appeared (or was preserved) in the evolution in the subspecies living in conditions of pronounced change of seasons, winter to summer, is of particular interest in the issue of interspecific differentiation. This feature is characteristic of subspecies of temperate climates, such as *A. m. mellifera* and *A. m. carnica*, but is absent in the subtropical subspecies *A. m. scutellata* and others [79, 95]. It was established that worker bees can accumulate levels of vitellogenin up to 50–60% of the hemolymph protein fraction because of limited brood raising [99, 110] and live for 6–10 months [87, 111].

Winter generation is characterized by a high life span and delayed organism aging, which is associated with influence of the protein vitellogenin and hormonal balance on the process of free radical oxidation. This feature allows the bee colony to survive the harsh long winter and to cope with many pathogens. An additional tension in the honeybee organism occurs during the long winter season in conditions of the northern part of the species habitat, for example, in Russia where the non-flight winter period lasts 5–7 months. During this time the intestines of the honeybee collect from 20 to 70 mg of undigested food residues. In this situation, the balance in the system ROS-antioxidants is especially important for the survival of the colony. First of all it relates to neutralizing putrefactive processes in the intestine. Otherwise, these residues are allocated by the bee inside the hive and can lead to the outbreak of noserosis and other diseases.

The European dark honeybee subspecies (*A. m. mellifera*) is the most evolutionarily adapted to northern conditions. Intensive studies of features of the honeybee adaptation to a long, cold winter conducted by MV Zhrebkin (1979) [112] showed that life span varies between bees appearing by (before) the warm season and focused on honey gathering during the entire plant vegetative period and bees appearing in the colony by the cold season for wintering provision. These differences are associated with the larval diet. The most winter-hardy subspecies, the European dark honeybee is characterized by a relatively large body mass of individuals and a greater volume of stored reserve nutrients, as well as a significant number of high-quality food stocks in the nest for successful wintering of the colony. The latter aspect largely determines the potential ability of different strains (types) of the honeybee to produce the total volume of honey in beekeeping. In the body of winter bees the amount of unstructured water gradually decreases and pharyngeal glands, ovaries, and fat body remain developed for a long time. Thus MV Zhrebkin showed that in the process of winter preparation the majority of bees in the colony acquired characteristics of physiologically young bees with

well-developed pharyngeal glands that produced large amounts of protein.

Subspecific differences include the specific functioning of the antioxidant defense systems in the gut and hemolymph of the honeybee and its distinctions in adaptation to climatic factors and exposure to the pathogens [66]. Comparative analysis in the climatic conditions of the Southern Urals revealed significant differences in the character of the stress reaction of bees of native subspecies *A. m. mellifera* and subspecies *A. m. caucasica*, introduced from the Black Sea coast. *A. m. mellifera* is characterized by a lower level of redox processes in the norm, a higher reactivity of the antioxidant enzymes, glucose-6-phosphate dehydrogenase and enzymes of PO cascade under the action of pathogens, but high and stable levels of glycosaminoglycans. This level of metabolic processes is justified in these natural climatic conditions, since a higher level of protective system response is necessary in case of threatened breach of homeostasis, ensuring response adequacy. A higher metabolic rate is constantly observed in introduced honeybees, which negatively affects the viability of the individuals if there appears excessive functional stress associated with any negative factor. These and other features of the protective reactions of *A. m. mellifera* reflect the stability of the European dark honeybee to climatic factors of the northern part of the species range [66].

Experimental data showed exhibition of normal peroxidase activity in *A. m. mellifera* honeybees predominantly in brain tissues, fat body, and the thorax muscles and mainly in the tissues of the midgut and large intestine in *A. m. caucasica* bees. Catalase activity in *A. m. mellifera* is much higher in the brain and hemolymph in comparison with other organs and tissues, and in the large intestine catalase activity increases only at the end of winter (prolonged nonflight period) in order to protect the bee organism from putrefactive processes in the intestine. This feature is also functionally fair for *A. m. mellifera*. In *A. m. caucasica* catalase activity is localized mainly in the tissues of the fat body and gut, organs that provide the highest level of metabolism [108]. This creates the conditions for maximum antioxidant protection, in particular, the catalase-peroxidase system [113, 114]. The high metabolic rate that is a characteristic of the southern subspecies as a whole will contribute to the emergence of stronger pathogenetic processes during development of stress reaction and reduce viability under the natural climatic conditions of the northern part of the species area.

During wintering not only behavior and metabolic rates of the honeybee individuals but also similar characteristics of the whole colony change, which is associated with changes in both external environmental factors

and the microclimate in the nest. Increase in the temperature in the nest during the winter is accompanied by a change in the ratio of CO₂ and O₂. In winter there is a transition of bees from aerobic to predominant anaerobic respiration in conditions of a high CO₂ content.

In conditions of reduced oxygenation, when the intensity of the Krebs cycle is reduced, in the mitochondrial respiratory chain substrates supplied by AOS (GSH, TRX, ascorbate) can be oxidized. Production of the alternative substrates of oxidation by AOS is conjugated with NADPH₂-generating mechanisms that are resistant to hypoxia and anoxia. A high activity of MnSOD is noted in the mitochondria. In intact cells in situ significant concentration of O₂^{•-} is not detected and leakage of electrons cannot serve as a sufficient ground for the destructive changes in mitochondria [115, 117]. In addition, in mitochondrial cycles the main constriction factors are catalase and enzymes with functions of GPX. Still-unknown functions of mitochondrial catalase and GPX-like enzymes have been proposed. These functions can be reduced to the transformation of H₂O₂ to O₂ and the subsequent intensification of respiration and ATP synthesis. Therefore, inhibition of catalase leads to a sharp decrease in the rate of respiration and oxidative phosphorylation in the mitochondria in vitro and in vivo. Flavoproteins possessing peroxidase activity play a major role in the process of linked oxidative phosphorylation [117]. TPX may also be such a flavoprotein, exhibiting peroxidase activity. The enzyme is multifunctional: It is involved in the peroxide metabolism of oxygen, restoring TRX and ascorbic acid and formation of deoxyribonucleotides in the metabolism of selenium-containing compounds. An important characteristic of the enzyme is its high resistance to respiratory toxins, which greatly distinguishes it from catalase and GPX [118].

20.5.1 Regulation of ROS in Honeybee Intestine

Features of climatic zones potentially affect honeybee behavior, physiology, and metabolism, as well as the development of interaction between the honeybee and pathogens [119]. In this regard, the intestine of the honeybee, which is one of the key points of the honeybee's interaction with its environment, is of great interest. In the first place, the intestine is the main organ of digestion, where nutrients and toxins, as well as enteric pathogens first come. The intestine of the honeybee in many respects determines resistance to disease at the level of individuals and the colony as a whole [120, 121]. The intestine was not randomly chosen as the main object for studying of the gene expression of different nature in the search for the causes of the Colony Collapse Disorder phenomenon [122].

TLR 3, Vanin-1, Ferretin 2, and chitinase expressed in the intestine and forming the basis of innate immunity in the honeybee differ in activity in different subspecies of the honeybee and determine the features of the confrontation of pathogenic microorganisms in different climatic conditions. Tool-like receptors are involved in the recognition of viral pathogens and also activate necrosis of the intestinal tissue under inflammatory reactions [123, 124]. Vanin-1 mediates oxidative burst in the intestinal epithelium, which occurs through the activation of the Tool/cytokine signaling pathway [125, 126]. Ferretin 2 also participates in the oxidative stress reactions; in this case it sequesters the excess ferric iron, reduces the generation of H₂O₂, and decreases the effects of oxidative stress in response to the activity of such proteins as Vanin-1 [127]. Chitinases are involved in the inflammatory response in the intestine induced by cytokines [128]. In addition, insect chitinases modulate the peritrophic membrane thickness, which forms a structure that separates the undigested nutrients from the intestinal epithelial cells [129].

The functioning of the basic biochemical mechanisms of the honeybee has been found to express caste and subspecific differences. Differential expression of the proteins involved in response to pathogens proves that various subspecies of the honeybee may have different levels of susceptibility to disease. These local adaptive responses may take place in the levels of protective protein expression, which may occur because of genetic or epigenetic changes in different subspecies of the honeybee. Metabolism in the honeybee subspecies is adapted to conditions in the place of their origin, so that the same climatic conditions have different effects on the biosynthesis, stability, and activity of proteins. All this contributes to the adaptation and conservation of the ecotypes. Therefore, measures should be taken for the study and preservation of species diversity of bees to prevent the loss of this rich genetic material that is so valuable to the world's biodiversity.

20.6 CONCLUSION AND PERSPECTIVES

The development of a unique ecological niche in the evolution of the honeybee defined a special role of oxidative stress in the vitality of the species.

One of the leading factors in the progressive evolution in the number of Hymenopteran species is the development of social instincts. In eusocial species there is a close contact with grown offspring, constant care of it at all stages of development. Communication means, maintenance of the optimal conditions of microclimate in the colony, and protection against enemies and adverse conditions have been significantly improved. Within

the colony there is a morphological and functional differentiation of individuals. In the individual actions of each individual the hereditary program that activates the mechanisms of organism alteration at certain times, physiological processes, and certain types of behavior plays a major role.

In the genome of the honeybee there are relatively few genes associated with innate immunity and detoxification proteins. In the evolution of the honeybee adaptation did not change the attributes of individuals but features of the bee colony as an integral biological unit. In accordance with this, the functioning of a large part of the genome is refocused on solving problems related to ensuring eusociality. Nevertheless, the reaction of phagocytosis in the hemolymph to pathogen introduction, the effect of a toxicant or other negative environmental impact, is accompanied by oxidative burst. It is traditionally appeared in the ROS generation and changes in antioxidant enzyme activity. Oxidative stress is a universal high-performance reaction of the honeybee organism to the impact of negative factors. Functional expression of hemocyte activation is the formation of quinone intermediates of melanin, $O_2^{\bullet-}$, H_2O_2 , and other ROS with high cytotoxic activity. Change in hormonal balance during the metamorphosis of the honeybee larvae and pupae and the appearance of macrophagocytes participating in processes of histolysis in the hemolymph are also accompanied by oxidative burst and induction of the molecular mechanisms to restrain it. Hormone-dependent changing of the functions performed by the working bee in the colony and control over longevity are also worthy of thorough study, including gerontology. Thus the honeybee is not only a subject economically and socially important to the human but also a very interesting model for studying of the mechanisms of memory, behavior, immunity, etc. In a complex system of protective reactions of the honeybee, oxidative stress and molecular mechanisms of its regulation demonstrate a high efficiency in solving a wide range of tasks with strictly limited resources. The honeybee genome [12] and the interpretation of its operating principles are expected to give a better understanding of the features of *A. mellifera* species.

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MOLECULAR BASIS OF IRON-INDUCED OXIDATIVE STRESS IN THE HONEYBEE BRAIN: A POTENTIAL MODEL SYSTEM OF OLFACTORY DYSFUNCTION IN NEUROLOGICAL DISEASES

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21.1 INTRODUCTION

It is well known that all living organisms are exposed to environmental stress. Many organisms are exposed to harsh environmental conditions such as extreme temperatures (freezing and heating), anoxia, desiccation, cross-tolerance, and oxidative stress, which may impair the operation of vital neuronal circuits and put animals in danger before these conditions directly cause cell and tissue death. Oxidative stress is defined as a condition that is produced by a disturbance in the cellular prooxidant-to-antioxidant ratio [1]. Reactive oxygen species (ROS) is a collective term that includes both oxygen radicals (such as superoxide, hydroxyl, peroxy, and hydroperoxyl radicals) and nonradical oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid, and ozone that can be converted into radicals. ROS are mainly produced by the mitochondrial electron transport chain and oxidation of polyunsaturated fatty acid (PUFA) [2].

In eukaryotic systems, reduction of molecular oxygen by one electron yields superoxide radical that has limited reactivity with some proteins but is not reactive with lipids or DNA (Fig. 21.1). Under the influence of superoxide dismutase (SOD), H_2O_2 is formed by the addition of 1 e^- and 2 H^+ . H_2O_2 is not a free radical because it does

not have an unpaired electron, but it is an effective nonradical oxidizing agent for many biological molecules because reduction of H_2O_2 yields hydroxyl radicals (OH^\bullet) [2]. Both superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl (OH^\bullet) radicals are continuously generated during oxidative metabolism in biological systems. $\text{O}_2^{\bullet-}$ reacts with some proteins; however, OH^\bullet radicals react with carbohydrates, proteins, lipids, and DNA (Fig. 21.1).

The level of oxidative stress is determined by the balance between the rate of induction of oxidative damage (depending on how fast ROS are generated) and the rate of efficient repair and/or removal processes (endogenous defense system including levels of repair enzymes and antioxidants) for such damage (Fig. 21.2). In response to low levels of ROS, the nuclear factor-erythroid-2-related factor 2 (Nrf2) translocates to the nucleus and regulates expression of genes involved in cell survival. At higher ROS concentrations, reaction between ROS and proteins or unsaturated lipids in the plasma membrane leads to a chemical cross-linking of membrane proteins and lipids and a reduction in membrane unsaturation, resulting in reduction of membrane fluidity and decreased activity of membrane-bound enzymes, ion channels, and receptors [3]. Furthermore, high ROS level prevents translocation of Nrf2 to the nucleus but stimulates nuclear factor- κB (NF- κB), a

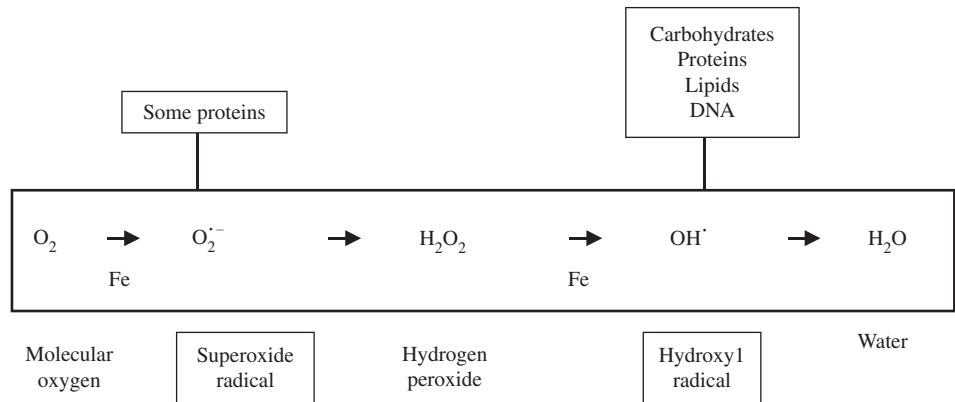


Fig. 21.1 Iron-induced reactive oxygen species (ROS) formation.

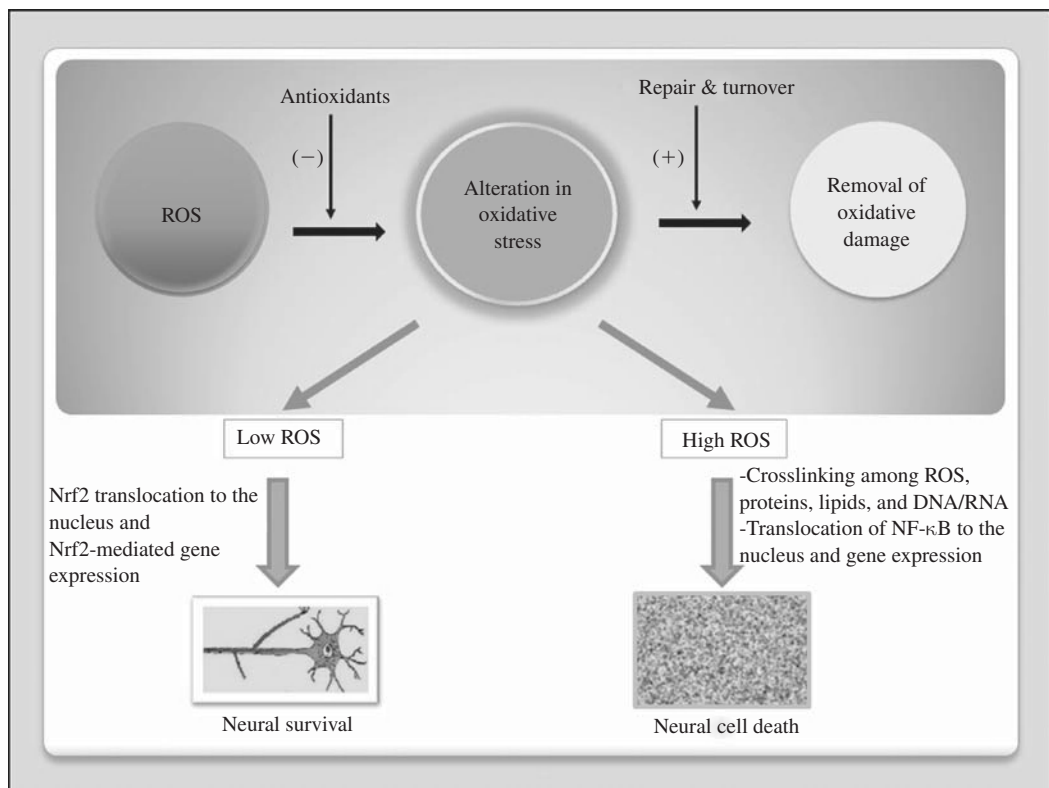


Fig. 21.2 Connection between reactive oxygen species (ROS)-mediated oxidative stress and abnormal functions. Increase in oxidant level, high ROS, decrease in antioxidants, and failure in repair and turnover result in functional abnormalities. Repair and turnover of oxidative damage, the so-called cellular defense mechanism, include DNA excision, resynthesis, and rejoining of DNA strands; repair of oxidized methionine residues in proteins; and normal membrane turnover releasing damaged lipids. Low ROS allow the translocation of nuclear factor-erythroid-2-related factor 2 (Nrf2) to the nucleus to regulate the expression of surviving genes for neural survival, whereas high ROS prevents translocation of Nrf2 to the nucleus. The cross-linking of high ROS occurs with proteins, lipids, DNA/RNA, which promotes neurodegeneration. (*See color insert.*)

transcription factor that, after translocating into the nucleus, induces expression of many genes involved in inflammation and oxidative stress and therefore promoting cell death (Fig. 21.2) [3]. The imbalance between

ROS production and cellular defense mechanism is also implicated in destruction of neural cells in a wide variety of pathological conditions such as abnormal functions, aging, and diseases.

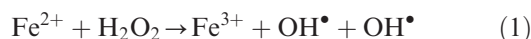
Iron, a transition metal ion, plays a crucial role in oxygen transport. Iron is a basic requirement for electron transport and cellular respiration and serves as a key element in most of the cytochrome enzymes involved in the oxidative phosphorylation of the Krebs cycle. Its overloading is potentially deleterious because of its involvement with lipid peroxidation in biological systems. Iron can induce generation of ROS through reduction of H_2O_2 to OH^\bullet via the iron-catalyzed Haber–Weiss/Fenton reactions [4, 5]:

Haber–Weiss reaction:



The Haber–Weiss cycle consists of the above two reactions. The Haber–Weiss reaction can form the hydroxyl radical (OH^\bullet) in an interaction between superoxide radical ($\text{O}_2^{\bullet-}$) and H_2O_2 in the presence of ferrous iron (Fe^{2+}) or ferric iron (Fe^{3+}).

Fenton reaction:



Fe^{2+} is oxidized by H_2O_2 to Fe^{3+} and produces the OH^\bullet and a hydroxyl anion in the Fenton reaction. Fe^{3+} is reduced back to Fe^{2+} , a peroxide radical ($\bullet\text{OHH}$), and a proton. The OH^\bullet is highly reactive. It can abstract a hydrogen atom from PUFA to initiate lipid peroxidation [6]. Increased Fe^{2+} directly initiates additional lipid peroxidation with accumulated lipid hydroperoxides, resulting in changes in membrane structure and functional damage [6]. The OH^\bullet attacks and damages every category of macromolecules such as lipids, proteins, carbohydrates, and DNA [7].

Brain is highly susceptible to oxidative damage. High rate of oxygen consumption, high lipid content, and relative scarcity in antioxidant enzymes compared to other tissues may account for the vulnerability in brain [8]. Several studies have demonstrated altered iron metabolism participating in the generation of ROS, resulting in marked increase in protein oxidation [9, 10], lipid peroxidation [11, 12], and DNA/RNA oxidation [13, 14] in the Alzheimer disease (AD) brain. Because of the excess of ferrous iron, oxidative stress has been shown to play a significant role in neurotoxicity associated with a variety of neurodegenerative and neuropsychiatric diseases (Table 21.1), affecting olfactory neuroepithelial cells at an early stage [15–33]. Increased oxidative stress is a prominent feature of vulnerable neurons in AD. Oxidative damage is shown to be increased in olfactory epithelium in biopsy

TABLE 21.1 Olfactory dysfunction frequently observed in patients suffering from brain diseases

Disease	Reference
Alzheimer disease	14–16
Alzheimer disease, Idiopathic Parkinson disease, parkinsonism-dementia complex of Guam	14–16, 18–21, 24, 29, 30
Huntington disease	22, 23
Amyotrophic lateral sclerosis	24, 25
Multiple sclerosis	26
Neuropsychiatric disorders	17
Schizophrenia	27, 28
Idiopathic REM sleep behavior disorder	30
Tauopathies	31
Open-angle glaucoma	32

specimens of AD [15]. ROS-mediated oxidative damage is markedly increased in olfactory epithelium from biopsy specimens of AD [15]. PAN-811 (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, or Triapine), a novel neuroprotectant, not only effectively suppresses intracellular ROS accumulation but also reduces ROS-mediated oxidative damage in both AD-derived and age-matched olfactory neuroepithelial cells [16]. It has also been shown that ROS-mediated olfactory deficit may be a characteristic feature of several neurological disorders including AD [15–17, 23], idiopathic Parkinson disease (PD), parkinsonism-dementia complex (PDC) of Guam [19, 20, 25], Huntington disease (HD) [23, 24], Guamanian amyotrophic lateral sclerosis (G-ALS), several forms of dementia [25, 30, 31], multiple sclerosis (MS) [27], schizophrenia [28, 29], idiopathic REM sleep behavior disorder [31], tauopathies [32], and open-angle glaucoma [33], supporting the view that there is a link among ROS, olfactory dysfunction, and neurodegenerative and neuropsychiatric diseases (Table 21.1).

Very little information is available on ROS-mediated oxidative stress in the insect brain. Although honeybees are a well-known model system for behavioral studies for olfactory learning and memory because they can be conditioned to respond with feeding movements of the mouthparts (proboscis) to a variety of floral odors, which is called proboscis extension reflex (PER) conditioning [34–37], only a few studies have been attempted to demonstrate a correlation between ROS-mediated oxidative stress in the brain and its effect on the learning behavior in individual restrained honeybees [38–40].

The purpose of the present overview is to discuss our iron-induced oxidative stress model system in honeybee *Apis mellifera* brain, created by injecting ferrous ammonium citrate (FAC) in the antennal lobes, which results in impairment of olfactory learning and memory [40] suggesting that oxidative stress plays a major role in

olfactory dysfunction. Olfactory deficits have been reported in many neurological disorders (Table 21.1). A similar mechanism has been proposed for olfactory abnormalities in patients of AD and PD. Because of the similarities in cellular and molecular processes that govern neuronal plasticity in humans and honeybees [38], the author proposes that the honeybee can be used as a potential and relatively simple model system for understanding human olfactory dysfunction in neurological diseases.

21.2 A COMPARISON BETWEEN GENERAL PHYSIOLOGY OF OLFACTORY PROCESSING IN HONEYBEES AND HUMANS

The honeybee *Apis mellifera* olfactory system is well adapted to detect and discriminate a diverse array of odors [41]. The perceptual qualities of odors vary depending on several factors such as carbon chain length in the drug, shape, functional group, as well as concentration [42–49]. The honeybee olfactory system possesses olfactory sensory neurons inside the cuticle-covered sensillae along the antennae, which are equivalent to olfactory epithelia within the nasal cavity in vertebrates [38, 50]. The olfactory receptors are located on the olfactory sensory neurons in antennae. The recognition and discrimination of odor molecules starts at the antenna via binding of odor with olfactory receptor. From the antenna, information is carried directly to the antennal lobe via axons of olfactory sensory neurons. The antennal lobe is the structural and functional analog of the olfactory bulb in vertebrates that is subdivided into identified glomeruli. It processes incoming signals from broadly tuned olfactory sensory neurons [51, 52]. The axons of olfactory sensory neurons converge on two types of antennal lobe neurons: (1) local interneurons communicate within the antennal lobe, and (2) outgoing projection neurons mediate the signal information from the antennal lobe to the protocerebrum [53]. The synaptic contact between olfactory sensory neurons, local interneurons, and outgoing projection neurons takes place within the glomeruli arranged in a single layer around the antennal lobe [54, 55]. The glomerular layer in the antennal lobe probably contains the summarized representation of the receptor types activated by a given odorant. Thus olfactory information received from the antennae is processed in the antennal lobe and relayed to the mushroom body and the lateral protocerebrum via projection neurons, leaving the antennal lobe in three different antenno-cerebral tracts. The small mediolateral antenno-cerebral tract contains pluriglomerular cells, whereas lateral and median antenno-cerebral tracts contain axons of uniglomerular projection neurons [55, 56]. A synaptic response to focal electrical stimulation recorded in the mushroom

body of the honeybee brain suggests involvement of the mushroom body in memory consolidation [57]. The synaptic plasticity exhibited in the mushroom body of the honeybee brain is similar to the synaptic plasticity occurring in the mammalian hippocampus [58]. Neuroplasticity in the honeybee brain, existing in the α lobe of the mushroom body, contributes to the associative long-term potentiation linked with olfactory learning and memory [59, 60]. This is in agreement with findings in *Drosophila* that all forms of olfactory learning (such as aversive, appetitive, and extinction) confined to the output sites of the mushroom body [61]. Moreover, GABAergic inhibitory interneurons in the antennal lobe of the honeybee brain modulate the overall activity and compute olfactory information by forming an odor-specific topographic map, relaying information from the antennal lobe to other brain regions via projection neurons [62–64].

Similar to the antennal lobe in honeybees, each glomerulus of the olfactory bulb in humans contains the axons of several thousands of olfactory sensory neurons, each expressing the same odorant receptor and the dendrites of mitral and tufted cells, which are the main input and output neurons of the olfactory bulb [65]. These neurons are activated by olfactory sensory neurons, but odorant information is further processed by lateral and feedback inhibitory pathways via activity of inhibitory interneurons, periglomerular cells, and granule cells [64–66]. During air inhalation, many volatile molecules reach the nasal cavity and interact with the odorant receptors located on the cilia of olfactory sensory neurons in the olfactory epithelium, eliciting an electrical signal that is transmitted to the second-order neurons in the olfactory bulb, which is then projected to pyramidal neurons in the olfactory cortex and to other brain regions such as thalamus and neocortex of the brain [38, 65]. Thus it is suggested that the peripheral olfactory systems involved in sensory transduction and early synaptic processing in the antenna and the antennal lobe of honeybees show a vast array of similarities to the olfactory epithelium and the olfactory bulb of humans [38, 68, 69].

21.3 OLFACTORY NEURONAL NETWORK IN HONEYBEES

In honeybees, the olfactory system is comprised of three major components: (1) antennae, (2) antennal lobes, and (3) mushroom bodies [38]. Honeybees use antennae to detect odors by making use of olfactory receptors that are located on olfactory sensory neurons in the antennae. Olfactory sensory neurons project their axons into the antennal lobes. The pathway involving olfactory sensory neurons is called the conditioned stimulus (CS) pathway, where CS represents an odor. Honeybees have

taste receptors located on olfactory sensory neurons in the antennae that project their axons into the antennal lobes. This pathway is called the unconditioned stimulus (US) pathway, where US represents nectar or sucrose. The primary sensory afferents (CS and US pathways) converge into the glomeruli of the antennal lobe.

In the honeybee *Apis mellifera*, an identified ventral unpaired medial cell (VUMmx1; an octopaminergic neuron) produces an associative link between US and CS pathways in the antennal lobe. The VUM interneuron releases octopamine into most if not all glomeruli of the antennal lobe innervating antennal lobe, mushroom body, and lateral protocerebrum. Electrical stimulation of VUM neuron substitutes for the unconditioned stimulus (sucrose) in an associative olfactory learning paradigm, supporting its role in olfactory learning and memory [70]. Local injection of octopamine into defined areas of brain increases honeybee's learning ability as well as recall, suggesting that octopamine is involved in regulation of learning and memory [71]. Actually, exactly how VUM interneuron couples the CS and US pathways is not clear except that this neuron has a reinforcing property. The convergence of two types of stimuli suggests that the antennal lobe is a site where part of olfactory memory consolidates [72]. The release of octopamine from VUM neuron or acetylcholine from olfactory sensory neuron in the synaptic cleft may induce GABA release from GABAergic local interneuron in the antennal lobe [38]. GABA-mediated inhibitory response is considered to be important for shaping and tuning the output from the antennal lobe [73].

21.4 ROS, OLFACTORY DYSFUNCTION, AND AGING

Increased oxidative stress is a prominent and early feature of aging and neurodegenerative diseases; therefore, oxidative damage has been considered an important factor in the progression of pathological and nonpathological age-related functional declines including olfaction. Again, this harmful condition occurs when there is either an excess of free radicals or a decrease in antioxidant levels, resulting in variety of changes in cells and tissues. Free radicals cause oxidative damage by attacking PUFA in neural cell membranes and generate metabolic waste products that interfere with cell-cell communication, damaging cellular proteins, lipids, and DNA, lowering energy levels and vitally impeding biochemical processes. The source of the devastating actions of ROS is mainly the oxygen molecule's unpaired electron, which makes it unstable and electrically charged [74]. Although sufficient ROS accumulation makes an organ system unable to function, endogenous defense

mechanisms can bring ROS levels back down to a normal level consistent with organ function. Increased levels of oxidative stress markers have been observed in cultured human olfactory neurons in AD patients compared to control subjects [75]. Oxidative stress in the aging murine olfactory bulb has shown significant changes in steady-state levels in olfactory bulbs of 20- versus 1.5-month-old mice, demonstrating greater carbonylation and nitration of specific proteins with aging [76]. Nitric oxide (NO) and derived nitrogen species in CNS may interact with catecholamines, thus modifying not only NO regulatory actions but also producing oxidants and free radicals that are likely to trigger toxic effects. Peroxynitrite, a potent oxidizing agent formed by the interaction between superoxide anion and nitric oxide, produces nitration of tyrosine groups present in the proteins and inactivates several enzymes [77]. Peroxynitrite appears to be involved in the pathophysiology of many neurodegenerative diseases. Increased oxidative stress in olfactory epithelium of AD patients causes modification in olfactory receptor proteins, resulting in olfactory impairment [78]. Moreover, abnormal processing of the proteins in AD supports contribution of RNA oxidation [14]. In humans, olfactory function deteriorates progressively with increasing age, even in the absence of overt medical problems [79]. Similarly, a marked deficit has been observed in olfactory learning and recall in healthy caged aged honeybee workers (30 days old) compared to young workers (4 days old), indicating that olfactory learning and memory processing in the honeybee brain appear to be vulnerable to aging [38]. Collective evidence supports the notion that ROS-mediated oxidative stress may have a strong impact on olfactory dysfunction with aging.

21.5 IRON INDUCES OXIDATIVE STRESS IN THE HONEYBEE BRAIN

Iron, an important ion used for biological electron transfer in aerobic systems, also exhibits a chemical dark side with side reactions that form ROS, resulting in oxidative stress. ROS-mediated lipid peroxidation has been suggested to be an important factor in posttraumatic neuronal degeneration [80]. Addition of FAC allows evaluation of a quick onset of oxidative stress that is free from other conditions, such as neuroinflammation, which are produced by chronic levels of iron injury during neurodegeneration [80, 81]. By applying *in vivo* injections of FAC into the antennal lobes of the honeybee brain to produce OH[•] radical, and monitoring the subject's responses to odorants by proboscis extension reflex conditioning assay, we have examined whether oxidative stress can be induced into the antennal lobes of the honeybee brain and whether or not ROS-associated alterations in brain can modulate olfactory

response of honeybees [40]. We have used different concentrations of FAC in each antennal lobe to evaluate its dose-dependent response and showed that the level of acquisition response in FAC-treated groups (0.5 mM to 5 mM range) significantly decreases with increasing dose (Fig. 21.3A), suggesting that iron interferes with olfactory learning in a dose-dependent manner. Testing of subjects in each group with conditioned odor (C), molecularly similar odor (S), and molecularly dissimilar odor (D) for retention and generalization responses show that iron interferes with retention in a dose-dependent manner but impairs generalization gradient at all FAC concentrations tested (Fig. 21.3B).

Reduced glutathione (GSH), an intracellular antioxidant, is known to prevent the oxidation of protein sulfhydryl groups and to help in maintaining cellular thiols in reduced state (-SH) [82]. To evaluate the reversal of FAC-mediated inhibitory effect on olfactory learning and memory, we injected GSH in each antennal lobe before injecting FAC, with an assumption that presence

of excess thiol compound in the brain may efficiently protect honeybees against toxic effects of ROS on olfactory learning and memory. We observed that the FAC-mediated inhibitory effect on acquisition response is fully reversed in presence of GSH (Fig. 21.4A). Pretreatment of antennal lobes with GSH before injection of FAC also results in complete reversal of iron-mediated inhibition of retention response as well as showing a characteristic generalization gradient (Fig. 21.4B). This suggests that increased concentration of antioxidant forms an important component of the defense by reversing the oxidative stress created by free radicals [40].

Several novel brain-permeant and multifunctional iron chelators (such as HLA20, M30, VK-28, and M32) have been tested for their ability to inhibit iron-dependent lipid peroxidation in rat brain homogenates [83]. The brain-permeant iron chelator VK-28 has been shown to prevent *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in mice [84] and 6-hydroxydopamine (6-OHDA) lesion in rats [85], without showing

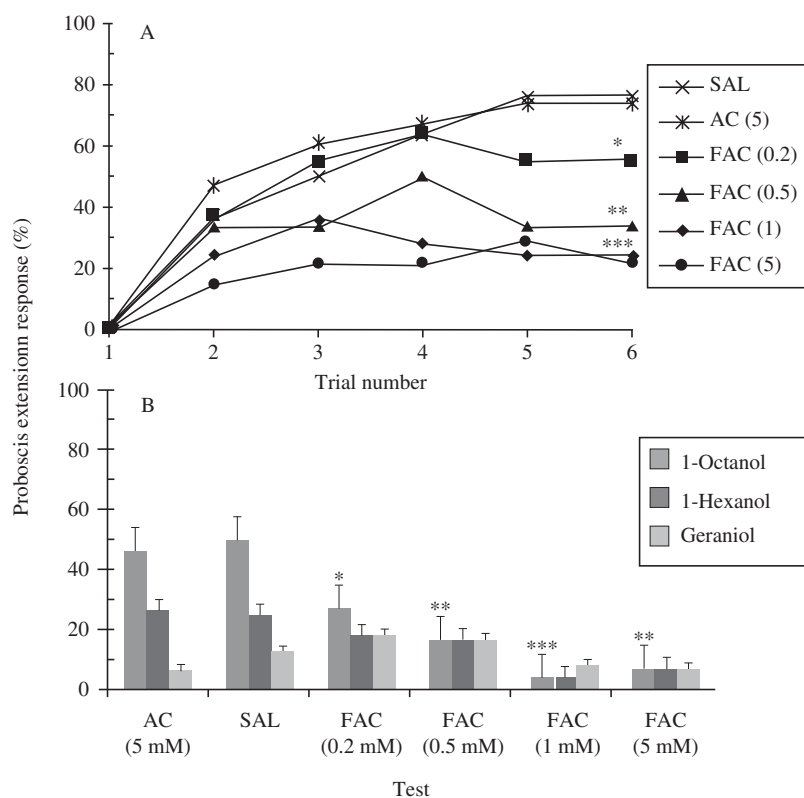


Fig. 21.3 Dose-response effect of ferrous ammonium citrate (FAC) on olfactory learning and memory. (A) Acquisition of the conditioned odor C: In this experiment, honeybees received 4 nl of saline (SAL), ammonium citrate (AC), or FAC at 0.2 mM, 0.5 mM, 1 mM, and 5 mM in each antennal lobe. Twenty-four hours later, subjects in each group were conditioned with 1-octanol (odor C). (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, molecularly similar (S), and molecularly dissimilar (D) odors in randomized order. *ns*, Not significantly different from controls (SAL, 5 mM AC). Asterisks indicate significant differences of respective points from control group: * $P = 0.05$, ** $P = 0.005$, 0.001, *** $P = 0.0001$). This figure is modified from reference [40]. (See color insert.)

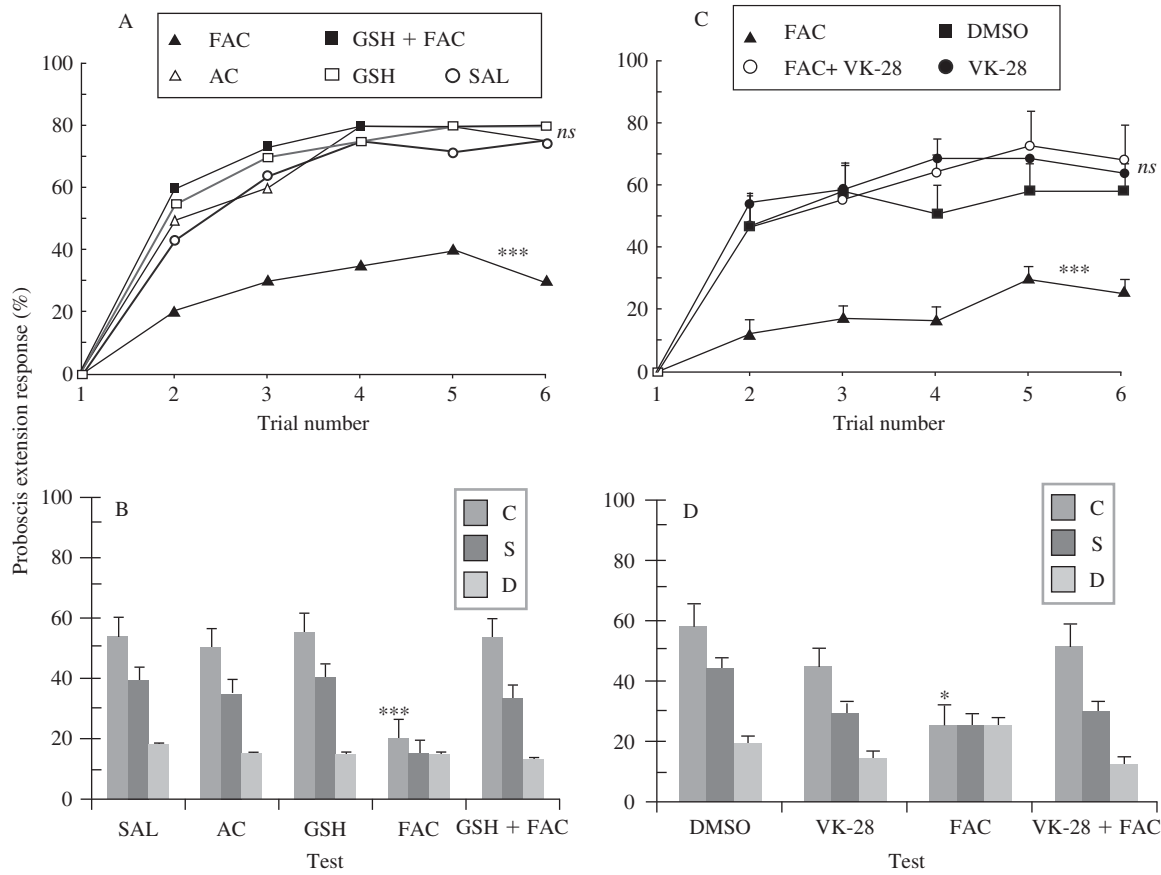


Fig. 21.4 Glutathione and VK-28 can reverse ferrous ammonium citrate (FAC)-mediated oxidative stress. (A) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either saline (SAL) or 2.5 mM reduced glutathione (GSH) in each antennal lobe. Two hours later, 4 nl of 500 μ mol/l ammonium citrate (AC) or 500 μ mol/l FAC were injected in each antennal lobe. Twenty-four hours later, subjects were conditioned with 1-octanol. (B) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with different odors (1-octanol, C; 1-hexanol, S; and geraniol, D) in randomized order. (C) Acquisition of the conditioned odor C: In this experiment, subjects received 4 nl of either dimethyl sulfoxide (DMSO) or VK-28 or DMSO + VK-28 in each antennal lobe 30 min before injection of FAC. Twenty-four hours later, subjects were conditioned with 1-octanol. (D) Test with odors C, S, D: Ninety minutes after conditioning, subjects in each group were tested with C, S, and D odors in randomized order. Asterisks indicate significant differences of respective points from control group (* $P = 0.05$, *** $P = 0.001$). *ns*, Not significant. This figure is modified from reference [40]. (See color insert.)

any appreciable inhibitory effect on monoamine oxidase (MAO) [86]. VK-28 inhibits both basal and Fe/ascorbate-induced mitochondrial membrane lipid peroxidation [85]. To confirm that FAC-mediated inhibitory effect on learning and memory is due to the addition of a high concentration of iron, we injected VK-28 into the antennal lobes of the honeybee brain before injecting FAC. We observed that the combination of FAC and VK-28 treatment restores learning response to 100% (Fig. 21.4C), implying that the FAC-mediated inhibitory effect on learning is due to increased ferrous iron. Subjects in the FAC-treated group show 40–50% reduction in retention response compared to controls and do not show a characteristic generalization gradient (Fig. 21.4D). However, FAC + VK-28-treated group overcomes

these inhibitory responses due to iron-chelating effect (Fig. 21.4D). Collectively, FAC-induced oxidative stress in the antennal lobes of the honeybee brain markedly inhibits olfactory learning and memory, supporting a major role of iron in olfactory dysfunction [40].

The inhibitory patterns of olfactory learning have also been observed by disruption of octopamine receptor function by either receptor antagonism or gene silencing [72]. However, addition of GSH into the antennal lobes prior to mianserin/dsRNA treatment does not reverse octopamine receptor disruption-mediated inhibitory responses of olfactory learning and memory [40], suggesting that octopamine receptor disruption and iron-mediated oxidative stress confer two independent mechanisms that impair olfactory learning and memory in honeybees.

21.6 MOLECULAR BASIS OF IRON-INDUCED OXIDATIVE STRESS

The brain is susceptible to iron-induced oxidative stress in both vertebrates and invertebrates. Oxidative stress in the olfactory system is a major factor associated with age- or disease-related olfactory impairment. Patients of AD at the early stage of the disease exhibit perceptual deficits in odor identification, although the molecular mechanisms are not completely understood. Protein oxidative modification in aging murine olfactory bulb has shown the presence of the specific carbonylated proteins in astrocytes and mitral/tufted neurons but nitrated proteins in the vasculature, as molecular substrates of age-related olfactory dysfunction [87]. Studies on redox-competent copper and iron indicate that redox activity in AD resides exclusively within the cytosol of vulnerable neurons, and chelation with deferoxamine or diethylenetriaminepentaacetic acid (DTPA) removes this activity [88].

The role of mitochondria as a potential source of redox-active metals and oxygen radical production is assuming more prominence. Increased mitochondrial DNA and cytochrome *c* oxidase activity but reduction in the number of mitochondria in AD brain indicates accelerated mitochondria turnover [15]. Neurons, and also the surrounding epithelial cells, in biopsy specimens have been observed with increased lipid peroxidation and heme oxygenase-1, whereas no increase in nucleic acid or protein oxidation was observed in vulnerable neurons in AD [15], suggesting that the abnormality in array of oxidative stress markers may occur depending on different cell types in AD.

A hypothetical scheme is shown in Fig. 21.5 to present molecular mechanism(s) involved with olfactory dysfunction in the honeybee brain. In the iron-induced oxidative stress model, phospholipase A_2 (PLA $_2$) peroxidizes PUFA that are present in phospholipids into peroxidized phospholipids, which are better substrates for phospholipase A_2 than native phospholipids in the honeybee brain (Fig. 21.5). This process results in the formation of free peroxidized arachidonic acid (ARA). The oxidation of ARA ultimately results in the generation of 4-hydroxynonenal, which impairs mitochondrial electron transport and produces ROS. Increased ROS production causes oxidative stress that damages proteins, DNA/RNA, and lipids, causing olfactory dysfunction (Fig. 21.5). Reduced glutathione protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins, whereas VK-28 chelates iron from the system and therefore slows down the formation of ROS. Oxidation of catecholamines may form quinones, which can form ROS, leading to olfactory dysfunction (Fig. 21.5). Furthermore, octopamine receptor function is disrupted by mianserin (octopamine receptor antagonist),

which competes with octopamine in binding to octopamine receptors and therefore blocks receptor function, resulting in olfactory dysfunction (Fig. 21.5). Octopamine receptor function disruption by octopamine receptor dsRNA (AmOA1-dsRNA) silences octopamine receptor gene expression, which inhibits its protein synthesis, resulting in olfactory dysfunction (Fig. 21.5). The receptor functional disruption mechanisms (mianserin/AmOA1-dsRNA) seem to be independent of ROS formation. The receptor functional disruption is also not a random process but appears to be associated with increased oxidation of specific proteins [89]. Similarly, disruption of noradrenergic receptor or production of dopamine quinones in the olfactory bulb of vertebrates (including humans) may contribute to increased levels of oxidative stress, resulting in olfactory dysfunction.

21.7 HONEYBEE MODEL SYSTEM FOR OLFACTORY DYSFUNCTION

The olfactory system is phylogenetically highly conserved among invertebrates and vertebrates. Honeybees have the ability to learn about the association of odor with reinforcement. There are well-established behavioral paradigms, such as proboscis extension reflex, for monitoring olfactory learning in honeybees. They have a short life span, which makes them suitable for study of aging. They are straightforward to propagate and easy to manipulate in the lab. They have short gestation periods that produce large numbers of offspring, and their genome sequence is available. Their brains can be easily dissected out to focus on the relevant neurons in a specific region such as antennal lobe from the honeybee brain because of its larger brain size compared to other insect models such as *Drosophila*. Finally, the antennal lobe of the honeybee brain is a functional analog to the mammalian olfactory bulb.

Oxidative damage has been demonstrated to be increased in the olfactory system (including neurons and the surrounding epithelial cells) in the brains of AD patients [15]. Neuropathological studies in the brains of PD patients also suggest neuronal damage in the olfactory system (including olfactory bulb and the anterior olfactory nucleus) [90]. These findings support our findings in the honeybee brain, where oxidative stress induced in the antennal lobe of the brain results in olfactory dysfunction [40]. All of the criteria mentioned above for a better model system for olfactory research, neuropathological studies performed in AD and PD brains [15,90], and our findings associating oxidative stress and olfactory dysfunction in the honeybee brain [38, 40] support the honeybee brain as a potential model system for olfactory dysfunction during aging and neurodegenerative diseases.

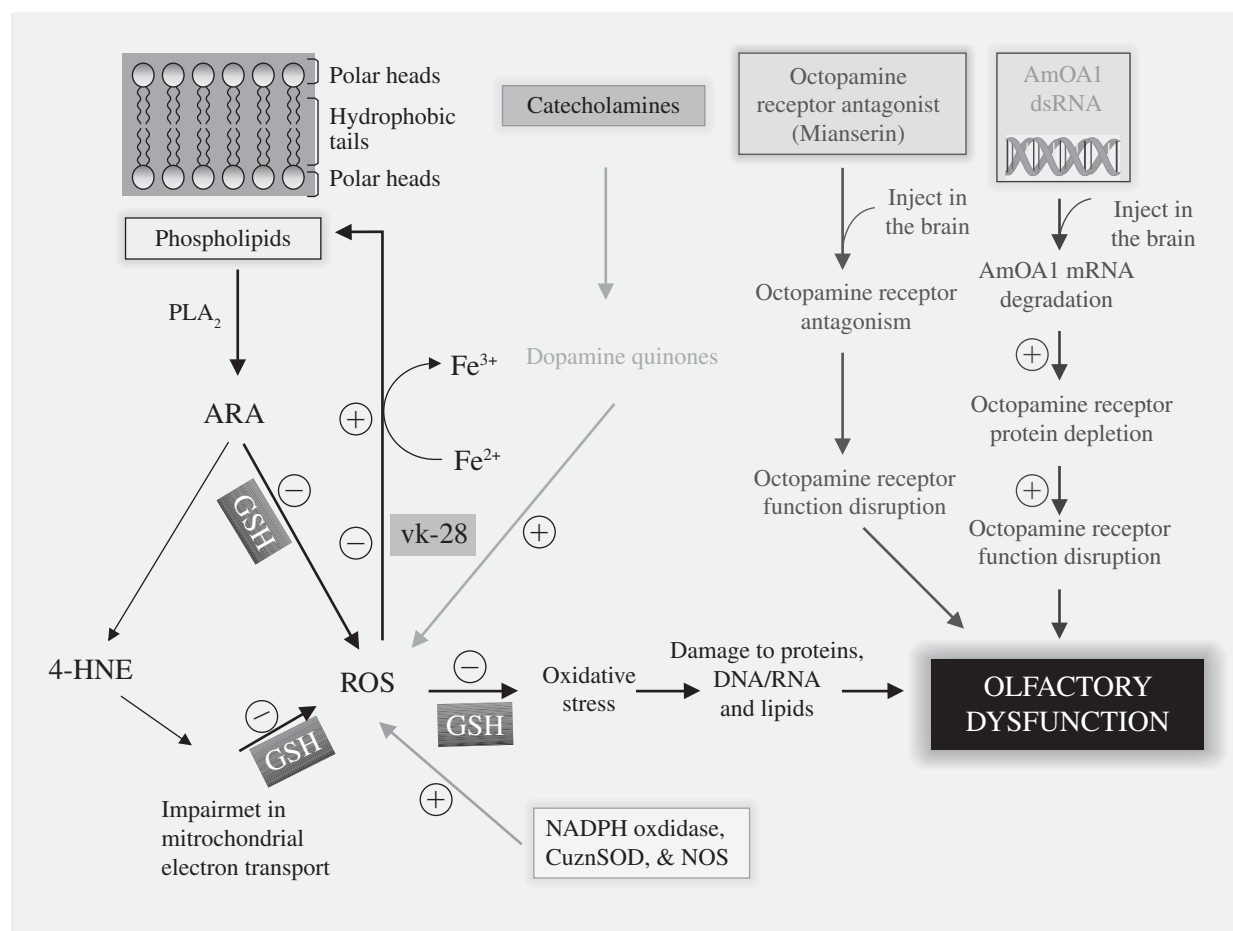


Fig. 21.5 Hypothetical molecular mechanism underlying reactive oxygen species (ROS)-mediated olfactory dysfunction in the honeybee brain. Iron-induced oxidative stress: Iron peroxidizes polyunsaturated fatty acids into peroxidized phospholipids, which are considered better substrates for phospholipase A_2 (PLA_2) than native phospholipids. PLA_2 catalyzes this reaction, forming arachidonic acid (ARA). The nonenzymatic oxidation of ARA results in the generation of 4-hydroxynonenal (4-HNE) that impairs mitochondrial electron transport, producing ROS. Increased ROS produces oxidative stress that damages proteins, DNA/RNA, and lipids, impairing olfactory processes (encoding, consolidation, and/or retrieval processes). Both octopamine receptor antagonism by mianserin (MAS) and octopamine receptor protein depletion by octopamine receptor double-stranded RNA (AmOA1-dsRNA) result in functional disruption of octopamine receptor, which leads to olfactory dysfunction. Oxidation of catecholamines forms quinones, which results in formation of ROS, leading to olfactory dysfunction. GSH protects brain against oxidative stress by modulating the redox state of specific thiol residues of target proteins. VK-28 chelates excess iron from the system. Monoamine oxidase inhibitor (MAOI) inhibits monoamine oxidase enzyme and therefore prevents oxidation of catecholamines. ROS can also be produced by activation of NADPH oxidase. (See color insert.)

21.8 CONCLUSION AND FUTURE PERSPECTIVE

Iron plays a role as a catalyst in free radical generation. Increase in ferrous iron catalyzes OH^\bullet radical production in the antennal lobes of the honeybee brain, which causes impairment in learning and memory. The inhibitory effect of iron-induced oxidative stress on learning and memory can be reversed by antioxidant as well as iron chelator in the antennal lobes, suggesting that the honeybee brain is highly susceptible to iron-induced oxidative damage and can be used as a model of olfactory dysfunction.

Iron is not regarded as the underlying cause of olfactory dysfunction in neurodegenerative diseases, but it does play an important role in progression of neurodegenerative diseases, such as AD and PD, by participating in redox reactions that catalyze the formation of ROS that produces increased oxidative stress, leading to olfactory abnormalities. Thus newly developed antioxidants and brain-permeant iron chelators (such as VK-28, HLA20, M30, and M32) can be screened for their rescuing effect on olfactory dysfunction in honeybees during aging and iron-induced oxidative stress. The efficacy of these drugs may depend on their ability to penetrate the subcellular

compartments and cellular membranes where iron-dependent free radicals are generated.

A reliable biomarker for oxidative stress is isoprostane, which is generated from cell membrane-bound ARA by free radical attack [91]. Isoprostanes have been described in vertebrate systems [92]. However, this information is not known in invertebrate systems. Therefore, another important challenge for future studies with the honeybee model system will be to determine levels of isoprostanes in the antennal lobes of FAC-treated and untreated brains, which can be used as an index of lipid peroxidation.

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MODULATION OF OXIDATIVE STRESS BY KEAP1/NRF2 SIGNALING IN *DROSOPHILA*: IMPLICATIONS FOR HUMAN DISEASES

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22.1 INTRODUCTION

The signaling module comprising the transcription factor Nrf2 (NFE2-related factor 2) and its cytoplasmic inhibitor Keap1 (Kelch ECH-associated protein 1) is a master mediator of ubiquitous antioxidant and detoxification responses to cellular stressors as well as of various cell type-specific homeostatic functions. Nrf2 belongs to the cap'n'collar (cnc) family of leucine zipper transcription factors named after the *cnc* locus of *Drosophila melanogaster*, which was originally characterized as a regulator of fly development. Even though the sequence similarity between Nrf2 and Cnc was evident early on, it took no less than a decade after Nrf2 was cloned to demonstrate experimentally its functional homology with a protein product of *cnc* (isoform C, CncC). This chapter recounts the story of these recent discoveries and discusses ongoing and future studies thereby made possible in flies on the Keap1/Nrf2 system as well as their implications for understanding and combating human diseases.

22.1.1 The Keap1/Nrf2 System Safeguards Homeostasis and Mediates Antioxidant Defense with Implications for Preventing and Treating Human Diseases

To defend themselves against the deleterious effects of oxidative stress, cells possess antioxidant defense networks that can sense prooxidant and electrophilic reactive

species and launch adaptive responses [1]. Since it was cloned in the mid-1990s [2], the vertebrate transcription factor Nrf2 has been established as a ubiquitously expressed master regulator of the cellular redox status and mediator of cell-protective antioxidant and detoxification responses [3–8]. Numerous studies have shown that in mice and in human cultured cells the expression of many protective genes increases in an Nrf2-dependent manner in response to oxidative and electrophilic chemical challenges [reviewed in 3, 4]. Target genes of Nrf2 include those encoding a broad range of redox regulators and so-called phase II detoxification enzymes, such as glutathione *S*-transferases, glutathione-synthesizing enzymes, thioredoxins, peroxiredoxins, NAD(P)H quinone oxidase 1 (NQO1), heme oxygenase 1 (HO1), and many others [9–12]. This battery of antioxidant and detoxifying genes are transcriptionally induced in a coordinated manner through the binding of Nrf2 to antioxidant response element (ARE) sequences in their regulatory regions (Fig. 22.1). The predominant dimerization partners of ARE-bound Nrf2 are the members of the small Maf (musculo-aponeurotic fibrosarcoma oncogene) family of proteins, which are themselves devoid of transcriptional activation potential [13–15].

The abundance and transcriptional activity of Nrf2 increase markedly when cells are exposed to oxidative stressors or electrophilic chemicals, and several of the mechanisms involved have been elucidated over the last

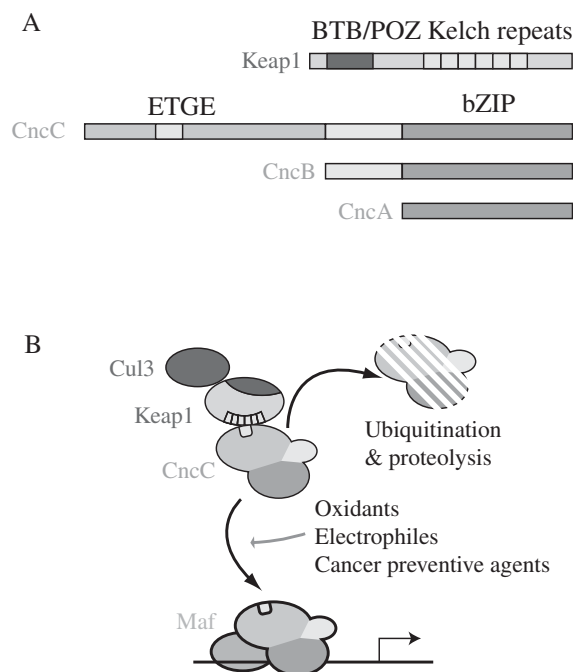


Fig. 22.1 Conservation and simplified illustration of the Keap1/Nrf2 pathway in *Drosophila*. (A) Nrf2 and Keap1 homologs are present in *Drosophila*. The fly *keap1* gene is predicted to encode a protein with high sequence similarity to its vertebrate Keap1 counterparts. Conserved domains include the BTB/POZ domain required for dimerization and 6 Kelch repeats for binding to Nrf2 and anchoring to actin. The *cnc* locus encodes three protein products, which all contain the bZIP region that mediates dimerization and DNA binding. The Nrf2 homolog is the longest isoform, CncC, which contains domains predicted to bind Keap1 such as the ETGE motif. (B) In basal conditions, Keap1 binds to CncC and inhibits its activity, likely through Cul3-mediated ubiquitination and proteasomal degradation. Oxidative stressors, electrophilic xenobiotics, and cancer chemopreventive agents relieve this inhibition. Stabilized CncC then accumulates in the nucleus and transcriptionally activates a battery of cell-protective genes, likely in a dimer with the single small Maf protein of *Drosophila*. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier. (See color insert.)

decade [reviewed in 16–19]. Notably, the activation of Nrf2 requires its posttranslational stabilization, because in nonstressed conditions Nrf2 undergoes rapid proteasomal degradation in the cytoplasm. This proteolysis is effected by a Cul3-based ubiquitin ligase system and facilitated by an interaction between Nrf2 and its cognate substrate adaptor, Keap1 (Kelch-like ECH-associating protein 1), a protein tethered to the actin cytoskeleton (Fig. 22.1) [3, 20–26]. The inhibitory effect on Nrf2 is abolished when specific redox-sensitive cysteine side chains of Keap1 undergo oxidative

modifications in response to oxidants and electrophiles, which likely triggers a conformational change in Keap1 structure that interferes with Nrf2 ubiquitination [27–32]. Additional mechanisms involved in Nrf2 activation include its phosphorylation by stress-activated kinases and the redox-sensitive regulation of its nuclear import and export [reviewed in 8, 16–8]. In addition to regulating adaptation to acute cellular stress via the induction of ubiquitous antioxidant and detoxification genes, the Keap1/Nrf2 system contributes to the homeostasis of diverse tissues by regulating the expression of corresponding sets of tissue-specific target genes with distinct roles in the specialized function of the respective cell types [reviewed in 4]. Its generic and tissue-specific homeostatic properties have established Nrf2 as an important “multiorgan protector” [4] and a mediator of “programmed cell life” [33].

By damaging proteins, lipids, and DNA, oxidative stress causes or exacerbates various diseases in model organisms and in humans [34, 35]. Conversely, by ameliorating oxidative stress or reversing its sequelae, Nrf2 protects against such pathologies. In mice, a protective role of Nrf2 has been demonstrated in diverse models of disease, including chemically induced cancers [3, 4, 6, 8, 36–39]; pulmonary diseases, such as asthma [40], emphysema [9], pulmonary fibrosis [41], hyperoxia [42], and acute lung injury [43]; neurodegenerative disorders, such as Parkinson disease [44], Alzheimer disease [45], and amyotrophic lateral sclerosis [46]; inflammatory disorders, such as inflammatory bowel disease [47]; liver toxicity, including toxin- and alcohol-induced liver damage [48, 49]; atherosclerosis [50]; and insulin resistance [51], among others [4, 6]. Importantly, compounds that activate Nrf2 have been shown to be effective in several of these disease models, highlighting Nrf2 as a promising target in preventing and treating various disorders [36, 52–57]. In humans, inherited DNA polymorphisms that reduce the expression of Nrf2 [58, 59] have been associated with diseases such as skin vitiligo [60], chronic gastritis [61], peptic ulcer [62], ulcerative colitis [63], and adult respiratory distress syndrome [59]. Moreover, impaired Nrf2 signaling has been implicated in human respiratory and neurodegenerative disorders [64–69]. Thus there is intense interest in elucidating the mechanisms by which Nrf2 activity is induced or impaired during the pathogenesis and progression of various human diseases, with the goal of identifying pharmacological or nutritional strategies to safely activate Nrf2 signaling for disease prevention and treatment. Although the model organism *Drosophila melanogaster* (commonly known as fruit fly) could help to obtain new insights relevant to these pursuits, it had not been utilized in the study of Nrf2 signaling until quite recently.

22.1.2 Cnc as a Developmental Locus of *Drosophila melanogaster*

Nrf2 is a member of the cap'n'collar (*cnc*) family of transcription factors, which are conserved from worms to mammals. Cnc factors are defined by the presence of a conserved 43-amino acid Cnc domain located amino-terminally to the DNA binding domain. In addition to Nrf2, the Cnc family comprises the vertebrate p45 NFE2 (nuclear factor erythroid-derived 2), which is present only in hematopoietic progenitor, erythroid, megakaryocytic, and mast cells and is required for proper development of platelets [70–72]; the NFE2-related factors Nrf1 and Nrf3 [73, 74], which have broad expression patterns partly overlapping that of Nrf2 and also function as stress-activated transcription factors [2, 73–75]; the *Caenorhabditis elegans* SKN-1 (Skinhead family member 1) [76], which is critical for the formation of the digestive system during worm embryogenesis [76] and also regulates a phase 2 detoxification response in the digestive tract [77]; and the *Drosophila* Cnc [78], after which the family was named.

The *Drosophila cnc* locus was originally identified 20 years ago as a gene expressed in the primordia of three segments of the developing fly embryo's head: the labral segment, where *cnc* expression is detectable as an anterior "cap," and the intercalary and mandibular primordia, where *cnc* expression is detectable as a more posterior 3–4 cell-wide "collar"—hence the name of the locus and of the gene family [78]. The anterior domain of *cnc* expression (corresponding to the labral segment primordium) was found to be activated by the *bicoid* and *torso* maternal pathways, independently of known zygotic gap genes, and sequentially constricted to its final size by repression from neighboring region-specific genes [79]. Control of the posterior domain (corresponding to the intercalary and mandibular segment primordia) involved combinatorial regulation by zygotic gap genes: activation by *buttonhead* and repression by *orthodenticle* anteriorly and *snail* ventrally [79]. Thus *cnc* is expressed and regulated as is typical for a second-tier region-specific gene. It was further shown that *cnc* is a homeotic gene that defines labral and mandibular segment identity: *cnc* mutants show absence of labral and mandibular head structures, and their mandibular tissues are transformed to a maxillary fate, which is a clear example of classical homeosis [80].

The *cnc* locus was subsequently found to give rise to three RNA isoforms, designated *cncA*, *cncB*, and *cncC*, each of which encodes a different protein [81]. CncA, CncB, and CncC share their carboxy-terminal regions, which include the DNA-binding domain, but have distinct amino-termini. CncC is the longest protein and encompasses CncB, which in turn encompasses the

shortest, CncA [81]. The isoform that mediates the role of *cnc* in embryonic head development was shown to be CncB: In mandibular cells, CncB antagonizes the ability of Deformed (Dfd), another homeotic transcription factor, to transcriptionally activate response elements of its downstream genes [81]. In the absence of CncB, unchecked Dfd activity transforms mandibular segment cells to cells of a maxillary fate [81]. A genetic interaction between *dfd* and *cnc* had been previously discovered in a genetic screen for modifiers of Dfd function [82], and these two genes were also found later to jointly regulate the initial embryonic expression pattern of the gene *proboscipedia*, a member of the Antennapedia complex that is required for the proper specification of the adult mouthparts of *Drosophila* [83].

In the specification of embryonic head structures, the CncB-mediated suppression of Dfd was found to require the *Drosophila* homolog of the mammalian small musculo-aponeurotic fibrosarcoma (Maf) proteins, named Maf-S [84]. In fly embryos, multimers of simple CncB/Maf-S heterodimer sites were transcriptionally activated in response to CncB, and in tissue culture cells the amino-terminal domain of CncB showed properties of a strong transcriptional activation domain [84]. Because the known elements that are activated in response to Dfd and are repressed in a CncB-dependent fashion do not match the CncB/Maf binding consensus sites, the suppressive effect of CncB/Maf-S proteins on Dfd activity must be exerted either indirectly or via direct binding to as yet uncharacterized Dfd response elements [84]. Similar to *Drosophila* CncB, the worm SKN-1 is required for the developmental specification of the pharynx, indicating phylogenetic conservation of this role of *cnc* transcription factors [76]. In contrast to *cncB* mutants, flies harboring mutations that selectively affect the *cncC* isoform develop normal larval head structures, indicating that CncC is not required for this developmental process [84]. However, *cncC* mutant flies are unable to complete larval development, suggesting a later developmental requirement for CncC [84]. The precise developmental role of CncC is an issue of substantial biological interest that awaits elucidation.

In addition to its role in head development, *cnc* is important for establishing the dorsoventral (DV) polarity of the oocyte's follicular epithelium during oogenesis [85]. Body axis establishment in *Drosophila* requires an ordered progression of events with determination of oocyte polarity, establishment of the anterior-posterior (AP) axis through localization of *bicoid* (*bcd*) and *oskar* (*osk*) mRNA at the anterior and posterior poles, respectively, and asymmetric movement and positioning of the oocyte nucleus in the anterior cortex, which defines the dorsal side of the egg chamber and initiates the DV patterning of the eggshell and the embryo. In developing

cnc mutant oocytes the nucleus is initially localized correctly at the anterior cortex. Progressively, however, the nucleus loses its proper localization; *osk* and *bcd* mRNA are mislocalized; microtubule arrangement is abnormal; and defects in DV polarity ensue [85]. Thus, whereas the early migration of the nucleus and organization of the oocyte microenvironment are independent of *cnc*, the stable anchoring of the nucleus to the anterior cortex of the oocyte and the proper specification of DV polarity require *cnc* function [85]. Because CncB is not expressed during oogenesis [81], the mutant phenotypes observed must be due to a lack of CncA, CncC, or both isoforms. Identifying the *cnc* factor(s) mediating establishment of DV polarity, and deciphering the underlying molecular mechanisms would contribute substantially to our understanding of the *cnc* locus and its functions.

22.1.3 Functional Conservation of Keap1/Nrf2 Signaling in *Drosophila*

Until a few years ago it had not been investigated whether Nrf2 shared its stress defense function with the founding member of the *cnc* family, *Drosophila* Cnc. In light of the intense interest in Nrf2 as a cell-protective factor in vertebrates and the fact that its *C. elegans* relative in the *cnc* family, SKN-1, had already been shown to have roles in antioxidant defense, the gap in knowledge regarding the family's namesake factor in *Drosophila* was perplexing. Nevertheless, even though no formal studies had been undertaken to evaluate the functional conservation of Nrf2 signaling in flies, both drosophilists and non-drosophilists had noticed that the *Drosophila* genome contained plausible homologs for *nrf2* and *keap1*. The CncC isoform was originally suggested as the likely *Drosophila* counterpart of Nrf2 because its unique amino-terminus showed sequence similarity to the Nrf2 domain that is required for binding to Keap1 [86]. The *Drosophila* genome was also found to harbor a likely homolog of vertebrate *keap1* genes known as CG3962, which had not been functionally characterized [86]. Other groups further documented the sequence conservation of putative Keap1-binding and transcription-activating domains in CncC, as well as the presence of ARE sequences in the upstream regions of *Drosophila* stress response genes [1, 87]. On the basis of these observations, it was proposed that CncC/Keap1 signaling is conserved in flies, with likely roles in the oxidative stress response and potentially also in development. As sometimes happens with good ideas in science, this one lingered for a few years without follow-up research studies to substantiate it. Thus the functional correspondence of *Drosophila* Keap1 and CncC to vertebrate Keap1 and Nrf2, respectively, remained an untested albeit plausible hypothesis.

Such was the state of the field at the time when we set out to formally test the idea that the *Drosophila* Keap1 and CncC comprise the fly Keap1/Nrf2 system. First, we found that *cncC* mRNA was most abundantly expressed in the alimentary canal of flies [88], which was reminiscent of the broad expression of Nrf2 in the digestive tract of mammals [89, 90]. *keap1* mRNA was also expressed in the alimentary canal, and both genes were also highly expressed in the Malpighian tubules, which are major sites of detoxification in flies corresponding functionally to vertebrate kidneys [88]. The expression of the candidate *Drosophila* homologs of Nrf2 and Keap1 in the digestive tract, which represents the first line of defense against ingested environmental stressors, and in the major detoxification organs supported the notion that *Drosophila* might employ Keap1/Nrf2 for stress defense, resembling vertebrates.

To test whether *Drosophila* Keap1 and CncC have functional homology to vertebrate Keap1 and Nrf2, we examined whether they can regulate the transcription of genes homologous to those regulated by Nrf2 in mammals. For this purpose we selected as candidate target genes *keap1* itself, which in vertebrates is regulated by Nrf2 in an autoregulatory loop [91], and *gstD1*, a prototypical oxidative stress response gene encoding a well-characterized detoxification enzyme [92], in whose upstream region we identified a sequence matching the ARE consensus [93]. Overexpression of CncC in flies was sufficient to substantially elevate the expression of *keap1* and *gstD1* mRNA [88]. Furthermore, *gstD1* and *keap1* levels were reduced by the conditional knockdown of CncC via RNA interference, and *gstD1* levels were increased by the conditional knockdown of Keap1. Consistently, *gstD1* levels were increased in flies heterozygous for either of the two *keap1* null (i.e., complete loss-of-function) mutations that we generated [88]. Taken together, these findings established *gstD1* and *keap1* as CncC-regulated genes, indicating functional homology of CncC with Nrf2.

To further address whether Keap1 and CncC mediate antioxidant and detoxification responses, we constructed transgenic reporter flies expressing green fluorescent protein (GFP) or β -galactosidase (*lacZ*) under the control of the ARE-containing genomic sequence upstream of the *gstD1* gene. In unstressed conditions fluorescence was detected most readily in the gut of *gstD-GFP* flies, consistent with the expression of *cncC* and *keap1* in this tissue [88]. Reporter activity was induced by CncC overexpression and by Keap1 knockdown in several tissues of flies at various stages of development. Using a genetic technique that is readily available in flies, we generated clones of cells in the brain that were homozygous for a *keap1* null allele, and found that the *gstD-lacZ* reporter was induced in these tissues. Moreover,

gstD-GFP reporter activity was induced when the flies were exposed to various oxidants, including hydrogen peroxide, the free radical generator Paraquat, the glutathione-depleting agent diethyl-maleate, and the heavy metal arsenic. In addition, the reporter was activated by the known Nrf2-activating compounds oltipraz and *tert*-butylhydroquinone. The knockdown of CncC suppressed *gstD-GFP* reporter inducibility by such conditions, and no induction was detected in transgenic reporter flies bearing a version of the enhancer in which the ARE had been mutated (*gstD Δ ARE-GFP*) [88].

To test whether an ARE sequence is sufficient to mediate transcriptional activation downstream of Keap1/CncC, we employed reporter flies containing a lacZ transgene driven by a synthetic sequence comprising a concatamer of binding sites that conformed to the consensus ARE and had been shown to respond to CncB (which has the same DNA binding site as CncC) [84]. Indeed, lacZ was markedly induced in CncC gain-of-function or Keap1 loss-of-function conditions, further supporting the functional conservation of ARE-mediated transcriptional responses in *Drosophila* [88]. Finally, we examined the contribution of Keap1 and CncC to oxidative stress resistance at the level of the intact organism. The conditional overexpression of CncC significantly increased the survival of flies that had been exposed to a semilethal dose of Paraquat; in contrast, CncC knockdown significantly decreased survival (Fig. 22.2) [88]. Taken together, these results demonstrated the functional homology of *Drosophila* Keap1 and CncC to vertebrate Keap1 and Nrf2, respectively. Much like their mammalian counterparts, *Drosophila* CncC and Keap1 regulate ARE-mediated transcription and detoxification gene expression and are crucial for the organism's defense against oxidative stress. Thus this work established *Drosophila* as a new genetically accessible model system for the study of this important homeostatic and cell survival pathway.

22.1.4 Advantages and Limitations of *Drosophila* as a Model Organism to Study the Nrf2 Pathway

22.1.4.1 Advantages For a century *Drosophila* has been one of the workhorses of genetic research [94, 95]. Therefore, the characterization of Keap1 and Nrf2 in this organism offers the possibility to use flies as a genetic platform for deriving new insights on the regulation and functions of this pathway. Before specific opportunities for such endeavors are presented, it is important to note that as a model organism *Drosophila* has several advantages over other models like worms, zebrafish, and mice. Naturally, it also has certain limitations. Although a general comparison of flies to other model systems is beyond the scope of this chapter, it is

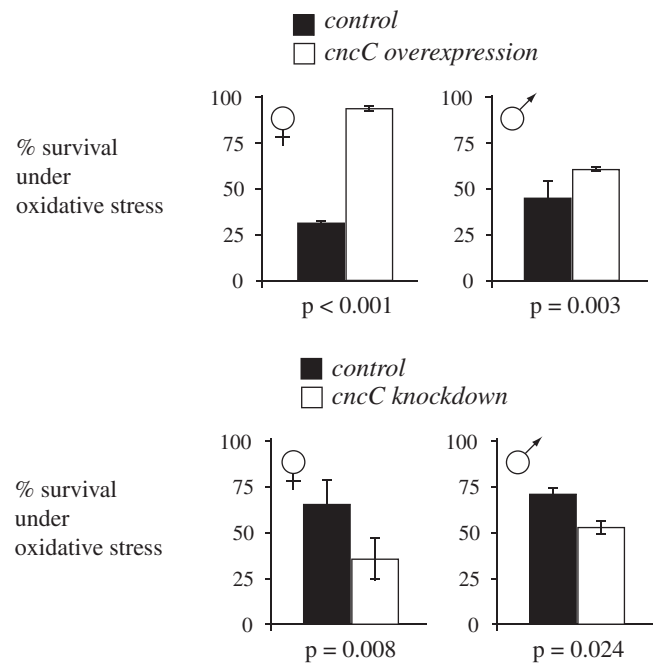


Fig. 22.2 CncC regulates oxidative stress resistance in *Drosophila*. (Top) Conditional ubiquitous overexpression of CncC before exposure to the free radical generator Paraquat significantly increases the flies' survival rate compared to genetically identical controls. Data are shown as means \pm SE of three experiments performed in triplicate. (Bottom) RNAi-mediated conditional ubiquitous knockdown of CncC before exposure to Paraquat significantly decreases the flies' survival rate. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

useful to highlight those advantages and limitations of flies that are relevant for the study of the Nrf2 pathway. Some of these features may be specific to the study subject. For example, the relatively short life span of the fly compared to mice makes it a very useful model for investigations of the genetic mechanisms that regulate aging and longevity, especially since such mechanisms are known to involve increased resistance to oxidative stress (see next section).

Other features of *Drosophila* that impact on its utility as a model organism for studies on Nrf2 signaling are directly related to its genetic properties. One major genetic feature of *Drosophila* is its low degree of genetic redundancy. Whereas higher organisms can have multiple genes encoding functionally related and even redundant proteins, this is rather uncommon in flies. At the same time, the large majority of human genes have known or presumed homologs in the fly genome. Thus flies strike a very useful balance of good conservation and low redundancy. This is true also in the case of the

Nrf2 pathway: Since CncB and CncA lack the Keap1-binding domain, CncC is assumed to be the only Nrf2 homolog. Flies, like mice and humans, have a single locus encoding Keap1, whereas worms have none and zebrafish have two [96]. Notably, the fly genome also carries only a single gene encoding a small Maf (CG9954), while none has been identified in worms; and anyway Skn-1 lacks the leucine zipper domain that mediates the dimerization of Nrf2 homologs in higher organisms with Mafs, and thus this aspect of Nrf2 function does not appear to be conserved in the worm [97]. Conversely, the small maf gene family is represented by no fewer than three members in vertebrates [98]. The genes encoding components of the Nrf2 ubiquitination and degradation machinery (Cul3, Rbx1, Cdn1, and others) are also well conserved in *Drosophila*. This combination of conservation with little genetic redundancy greatly facilitates investigations of Nrf2 signaling in flies, including studies that might be impossible or very difficult in organisms with either an incomplete Nrf2 module or a high degree of redundancy. At the same time, this property of *Drosophila* suggests that the results of fly studies might be readily extrapolated to higher organisms, in which the pathway is similarly constructed, to form the basis for experimental validation of insights derived from flies.

Another major advantage of *Drosophila* is its exquisite amenability to genetic manipulation. Various genetic technologies have been developed that allow the up- or downregulation, inactivation, or ectopic expression of a chosen gene either ubiquitously or in a tissue-specific and/or temporally controlled manner. With these methods, *Drosophila* offers increased efficiency, higher speed, and lower cost of experimentation compared to vertebrates. Thus, now that the principal role of CncC in oxidative stress protection of *Drosophila* has been established, flies can be used to investigate the tissue-specific requirement for Nrf2 signaling in defending various organs and sustaining their specialized functions [4]. Moreover, the phylogenetic conservation of Nrf2 signaling in flies offers the opportunity to identify new genes that regulate the pathway through unbiased genetic screens. The critical requirement for conducting such screens is to establish an appropriately sensitive *in vivo* or cell-based assay reflecting the activity status of the pathway, such as the one shown in Fig. 22.3. It then becomes possible to identify pathway components and modulators either *in vivo*—by utilizing available large collections of flies harboring chemically induced mutations, transposable elements disrupting gene function, or transgenes expressing gene-specific double-stranded RNAs (dsRNAs) that trigger gene knockdown via

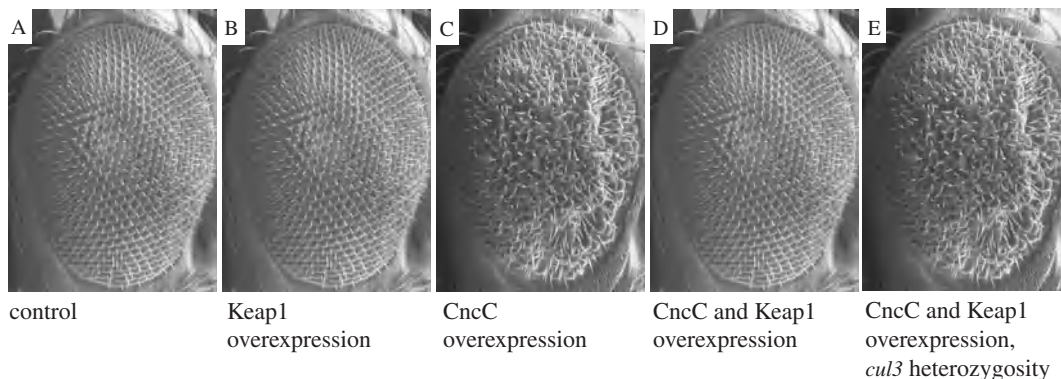


Fig. 22.3 A genetically sensitized *in vivo* *Drosophila* assay of Keap1/Nrf2 system activity. (A) The compound eye of *Drosophila* comprises about 800 individual ommatidia (meaning “small eyes”) arranged in a regular pattern. This pattern can be disrupted by the genetic manipulation of cell signaling during eye development, generally without causing lethality. Thus the *Drosophila* eye is a convenient system for the analysis of regulatory interactions between genes. (B) The wild-type pattern is unaffected by the eye-specific overexpression of Keap1. (C) In contrast, eye-specific overexpression of CncC results in a rough-appearing eye due to loss and disrupted arrangement of ommatidia. (D) Coexpression of Keap1 and CncC results in wild-type eye appearance. The complete suppression of the CncC-induced phenotype by Keap1 is consistent with its role as an inhibitor of Nrf2 signaling. Thus the interaction of overexpressed Keap1 and CncC in the *Drosophila* eye is an *in vivo* assay reflecting Nrf2 pathway activity. (E) The eye interaction of Keap1 and CncC is sensitive to genetic manipulation, as demonstrated by the reemergence of the rough eye phenotype in the presence of either one of two *cul3* alleles (the chemically induced mutation *cul3*², and the transposable element-induced mutation *cul3*⁰⁶⁴³⁰). Therefore, this eye interaction assay, or other similarly sensitized *in vivo* or cell-based readouts, could be used to test whether specific candidate genes are regulators or components of the Nrf2 pathway, as well as to identify new such genes through unbiased genetic screens. This figure is adapted from Sykiotis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

RNA interference (RNAi)—or in cultured cells—by screening whole-genome collections of gene-specific dsRNAs. Such screens are, of course, also feasible in *C. elegans* and in cultured mammalian cells, and they are currently being pursued in those systems as well to dissect the Nrf2 pathway. The experimental facility of *Drosophila* stems again from its combination of good conservation and low redundancy. This increases the power to identify as screen “hits” genes that may be absent in worms and/or may escape detection in mammalian cells because of mutual compensation among redundant members of the same gene family. The relative ease with which secondary screens can be conducted to validate and characterize such hits is another advantage of the *Drosophila* system. Thus fly screens aiming to expand the CncC pathway should be vigorously pursued and should be cross-referenced to the few worm RNAi screens on SKN-1 that have been published [99–101], as well as to future worm and mammalian screens. Previously unknown pathway modulators discovered in this manner may represent favorable targets for the ubiquitous or tissue-specific pharmacological modulation of Nrf2.

22.1.4.2 Limitations Before embarking on fly studies focused on the Nrf2 pathway, one should also bear in mind certain limitations of the experimental system. One such feature is that null mutations in each of the core pathway components lead to lethality during development. Not only *cnc* isoform-nonspecific but also *cncC*-specific null mutations are lethal [84]. This is in contrast to mice, where *nrf2*^{−/−} animals are viable and fertile [89]. Lethality can be a useful phenotype for developmental studies, where it can facilitate the discovery of novel molecular mechanisms as well as interacting genes. However, it is an obvious disadvantage for studies of homeostasis and responses to external challenges in the mature organism. In our studies, we circumvented this problem by using a conditional RNAi-mediated knock-down of CncC in adult flies to test for effects on oxidative stress tolerance [88]. Nevertheless, such methods are unlikely to completely eliminate gene expression and to phenocopy null mutations, and thus probably underestimate the magnitude of CncC-mediated effects. The drosophilist’s quiver holds several other arrows to hit these targets, including the isolation of temperature-sensitive mutations that behave as nulls, hypomorphs, or amorphs depending on the temperature at which the flies are reared, or the rescue of the developmental lethality of a null allele by tissue-specific transgenic expression of a corresponding wild-type cDNA construct. The application of such methods, however, would be substantially informed by first elucidating the reasons for the developmental lethality of *cncC*-specific alleles. Since SKN-1 is critical to the formation of the digestive system during

worm embryogenesis [76], the developmental lethality of CncC during the larval stage might also be related to a requirement for proper development or function of the gut, where *cncC* mRNA is highly expressed [88]. Similarly to *cncC*, loss of both *keap1* alleles leads to lethality during larval development [88]. The lethality of *keap1* null alleles may be due to unchecked induction of CncC, because, whereas conditional moderate overexpression of CncC can confer oxidative stress resistance, its constitutive high overexpression can be lethal to both individual cells as well as the organism [88]. Unfortunately, the developmental lethality of *cncC* mutations [84] precludes a straightforward genetic test of this hypothesis.

In addition to its genetics, the anatomy and biology of *Drosophila* each place limitations on the use of flies to study the Nrf2 system. Even though the ability to manipulate gene expression in flies in a tissue-specific manner could help to investigate some of the tissue-specific functions of Nrf2 signaling, the small size of flies can make it technically challenging, though not necessarily impossible, to evaluate some of these potential functions. Such difficulties may be encountered when examining, for example, dopaminergic neurons in studies on neuronal degeneration (see next section) or oenocytes, the equivalent of vertebrate liver cells, in potential studies on metabolism. For studies that involve the administration of Nrf2-activating compounds (or, potentially, Nrf2-inhibiting compounds), the choice of an appropriate solvent can become a challenging problem. The most commonly used solvent both for maintaining large-scale compound libraries and for dissolving individual compounds is dimethyl sulfoxide (DMSO). Unfortunately, DMSO is an oxidant [102] and may be deleterious in high concentrations. Intact flies are generally assumed to tolerate concentrations of DMSO in their diet of up to 1%. However, it has been shown that even 0.5% of DMSO in the food may lead to developmental, reproductive, and cellular toxicity [103]. This observation cautions against the assumption that “low” concentrations of DMSO are innocuous. Consistently, we have found that DMSO concentrations as low as 0.1% can interfere in vivo with readouts that depend on CncC activity (Fig. 22.4). Thus, if DMSO is used as a solvent in experiments focused on the Nrf2 pathway in flies, caution must be exercised in the interpretation of the experimental results. Specifically, if the administration of CncC-activating compounds dissolved in DMSO is observed to exert beneficial effects against a toxic insult or other detrimental condition compared to the administration of the DMSO solvent alone, this could in principle reflect (partly or wholly) a rescue of DMSO’s toxicity or of an additive/synergistic toxic effect of the two oxidants (DMSO and the insult under study).

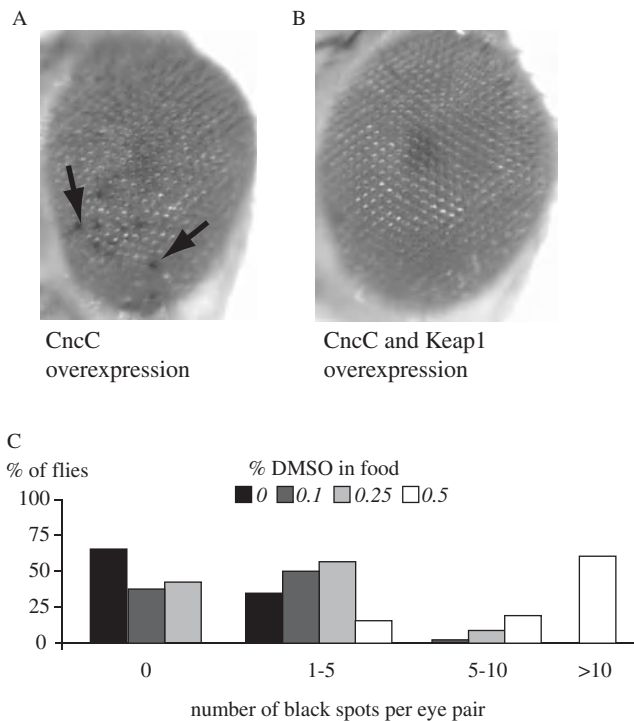


Fig. 22.4 The commonly used solvent DMSO can affect Nrf2 pathway activity in *Drosophila*. (A) In addition to missing and disarranged ommatidia, the eyes of flies overexpressing CncC show black spots (indicated by arrows) that probably represent necrotic and/or melanotic ommatidia. The number of these spots per eye pair correlates with the severity of the phenotype. (B) When Keap1 is coexpressed with CncC in the eye and the flies develop in normal food, the presence of black spots in the eye is suppressed, consistent with the inhibitory function of Keap1. (C) When flies coexpressing CncC and Keap1 in the eye develop in food containing DMSO, which is an oxidant, black spots are detected, and their number correlates positively with the concentration of DMSO. More than 300 flies were scored at each DMSO concentration.

Ethanol may be a more appropriate solvent in such cases, at least in cultured cells, as it most likely evaporates from the medium that flies grow in/on. We have also found lipid-based solvents to be problematic because of their high viscosity: As they are excreted from the flies' guts on the container walls, they tend to trap adult flies—such solvents may, however, be suitable for studies on larvae.

Finally, there is no escaping the fact that *Drosophila* is a latecomer to the Nrf2 arena, in which mammalian studies have been ongoing for more than a decade and worm studies for several years. Thus the cost-benefit ratio of investing in flies to address a research question on the Nrf2 system should be carefully weighed. The study topics ought to be carefully selected to take advantage of *Drosophila*'s unique assets and relative strengths, so as to facilitate real breakthroughs and not mere replications or extensions of mammalian studies. Thus a high degree of strategic planning will be required over the short term to “catch up” with worms and vertebrates in characterizing the pathway, while at the same time proving the value of the fly system for Nrf2 research by deriving unique insights not readily available from these historically leading experimental platforms. Table 22.1 lists some of the fundamental questions that should be addressed as soon as possible to better characterize the pathway in flies, and highlights research directions that should be pursued in parallel because they have the potential to lead to contributions of major impact in the field of Nrf2 biology.

22.1.5 New Insights Derived from *Drosophila* on the Functions of the Nrf2 System

By establishing the phylogenetic conservation of Nrf2 signaling in *Drosophila* and exploiting the experimental

TABLE 22.1 Prospects for Nrf2-related research in the *Drosophila* system.

Fundamental questions that need to be addressed to better characterize the Keap1/Nrf2 system in <i>Drosophila</i>	Questions of Nrf2 biology for which <i>Drosophila</i> could be used to derive novel insights of potentially major impact
<ul style="list-style-type: none"> • Is the single small Maf of <i>Drosophila</i> the interaction partner of CncC in activating transcription via the ARE? • Do CncA and CncB have roles in regulating ARE-mediated protective responses? Is CncA, which lacks transcriptional activity, an ARE repressor? Is CncB, which lacks a Keap1-binding domain, a constitutive ARE activator? • Is CncC degraded via the action of Cul3, similarly to vertebrate Nrf2? • Which is the set of CncC target genes, and does it include genes with tissue-specific homeostatic roles like vertebrate Nrf2? • Which are the naturally-occurring oxidative stressors that CncC has evolved to protect flies against? • What are the reasons for the developmental lethality of flies homozygous for <i>cncC</i> and <i>keap1</i> mutant alleles? 	<ul style="list-style-type: none"> • Which genes are required for the activation of Nrf2 signaling by oxidants and other inducers? • Which genes encode targets for the pharmacological activation or inhibition of Nrf2 signaling? • By which mechanism(s) does the relative abundance of ARE-binding factors determine the transcriptional status of Nrf2 target genes? • How is Nrf2 signaling involved in promoting longevity and extending the healthy life span? • How does Nrf2 prevent the degeneration of dopaminergic neurons in vivo? • What are the mechanisms by which Nrf2 affects and is affected by the metabolism of sugars and lipids?

advantages of flies, new insights have already been gleaned on the functions of the Nrf2 system; albeit few in number, these advances are of substantial impact, as they pertain to the roles of Nrf2 in the regulation of longevity [88], the degeneration of dopaminergic neurons in Parkinson disease [104], and the resistance to the environmental pollutant methylmercury [105].

22.1.5.1 The Keap1/Nrf2 Pathway as a System Controlling Longevity One of the most interesting biological questions is whether and how the aging process is regulated and how longevity and youthfulness might be extended. According to the oxidative stress theory of aging, oxidative damage to biological macromolecules is a key driver of aging; conversely, delaying the accumulation of oxidation products in the cells and tissues of an organism could promote longevity [106]. Studies in model organisms have supported this hypothesis by showing that life span extension by various genetic or dietary manipulations correlates with increased oxidative stress resistance, as well as that the overexpression of antioxidant genes can not only augment oxidative stress tolerance but can also promote longevity [107–110]. As a master regulator of antioxidant and detoxification responses, Nrf2 was a plausible regulator of the aging process. Indeed, SKN-1 was shown to be required not only for normal life span in *C.elegans* [77] but also for the life span-extending function of caloric restriction through its function in a pair of specialized neuroendocrine cells in the worms' brain [111]. Having characterized the Nrf2 system in flies, we undertook a study to address whether activation of Nrf2 signaling could promote longevity under normal laboratory growth conditions [88].

For this test we employed flies heterozygous for either of two independent *keap1* alleles. These flies showed elevated mRNA levels of the CncC target gene *gstD1* (Fig. 22.5), suggesting that loss of one copy of the *keap1* gene can result in a gain of CncC function in vivo [88]. Male heterozygous *keap1* mutant flies showed increased resistance to Paraquat and also had a median life span significantly longer than that of their otherwise genetically identical siblings (Fig. 22.5) [88]. These findings demonstrated that partial loss of function of the Nrf2 pathway's negative regulator can have significant beneficial effects on the oxidative stress tolerance and longevity of male *Drosophila*. This was consistent with the oxidative stress hypothesis of aging and also provided the first evidence for a role of Keap1 in life span regulation. Interestingly, female *keap1* heterozygotes did not show significant differences in either Paraquat resistance or longevity (Fig. 22.5). Although we have not yet thoroughly investigated the underlying reasons for this sexual dimorphism, we suspect that it reflects

hormonal or metabolic differences between the sexes. A subsequently published study in *C. elegans* showed that SKN-1 mediates the life span extension associated with reduced insulin/IGF-like signaling and that its transgenic overexpression at certain levels can also confer longevity [112]. Taken together, these studies in invertebrates revealed a new role for Nrf2 signaling as an antiaging and life span-extending factor that warrants detailed characterization in invertebrate and vertebrate models [further discussed in 8].

22.1.5.2 Protective Role of Nrf2 Against Parkinson Disease Oxidative stress in the nervous system is associated with neuronal cell death during the pathogenesis of multiple neurodegenerative disorders, including Parkinson disease (PD) [113, 114]. PD is an age-related disorder in which severe oxidative damage occurs in the substantia nigra, resulting in the degeneration and loss of dopaminergic (DA) neurons [115]; this manifests clinically as progressive functional impairment with ultimate mortality. Effective treatments for PD are lacking, and thus a better understanding of the mechanisms regulating DA neuron survival and death is urgently warranted. A series of recent studies have implicated the Nrf2 signaling pathway as an important factor for DA neuron survival during PD pathogenesis. Notably, compounds that are approved treatments for PD, such as bromocryptine and selegiline, were found to activate Nrf2 [116, 117]. Genetic and pharmacological studies also documented the importance of Nrf2 as a protective factor against PD in mouse models of the disease in which DA neuronal toxicity is elicited through administration of the prooxidant compounds 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) [44, 118–120]. Furthermore, a recent human study documented that the few surviving DA neurons of postmortem brains from PD patients showed increased abundance and nuclear localization of Nrf2 [68]. Consistently, other studies have shown that the expression or function of p62 and DJ-1, which are both proteins modulating Nrf2, is compromised in neurodegeneration, likely leading to impaired antioxidant response. p62 is a cytoplasmic protein that mediates the formation of ubiquitinated aggregates that are removed by autophagy [121, 122], and it is localized to intracellular aggregates in various neurodegenerative diseases [123]. p62 binds directly to Keap1 and increases the protein stability of Nrf2 [124–127]; moreover, Nrf2 upregulates the transcription of *p62* [126, 128–130]. The *p62* promoter shows increased oxidation in PD leading to reduced *p62* gene transcription, which may account for Nrf2 dysfunction [131]. Regarding DJ-1, inherited loss-of-function mutations in its gene (*PARK7*) are associated with early-onset

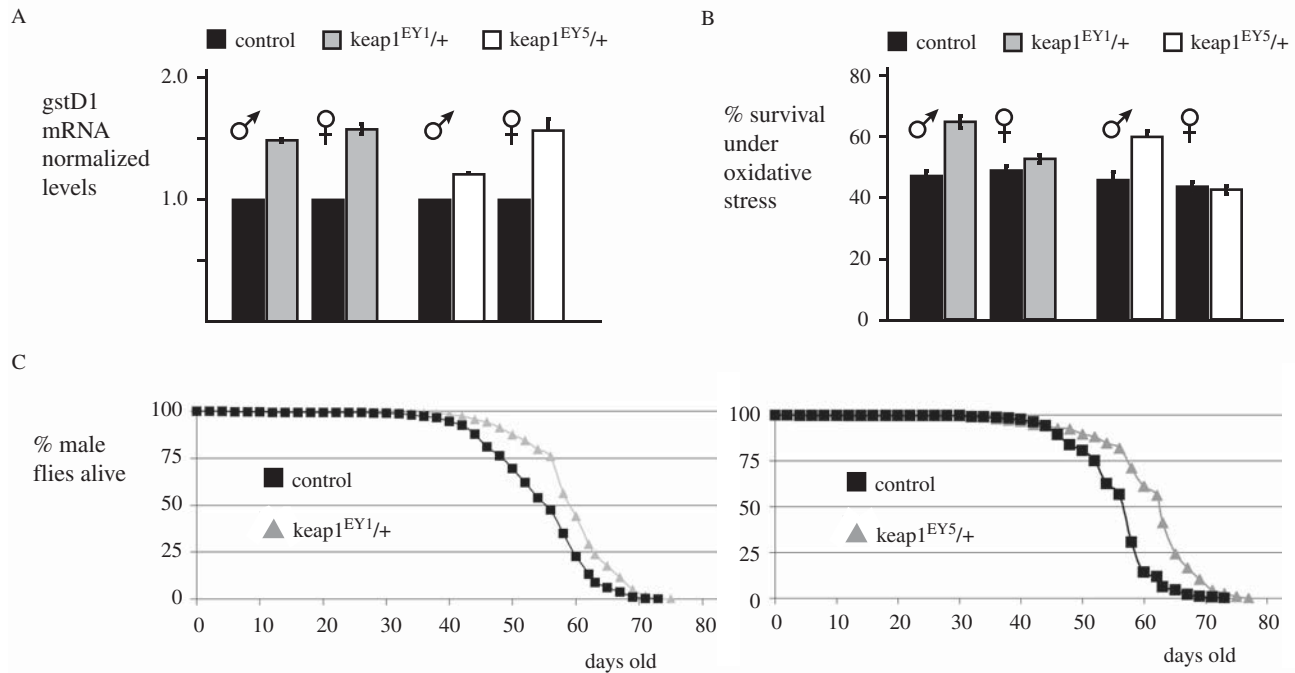


Fig. 22.5 Heterozygosity for *keap1* is associated with increased oxidative stress resistance and extended longevity in male *Drosophila*. (A) The *keap1^{EY1}* and *keap1^{EY5}* alleles were created by the reinsertions of mobilized transposable elements in the coding sequence of the *keap1* gene. Both alleles cause larval lethality and are presumed nulls. One-day-old male and female *keap1* heterozygous flies have significantly elevated *gstD1* mRNA expression levels (normalized to *actin* mRNA levels) as quantified by real-time RT-PCR. Control flies were siblings of the *keap1* heterozygotes and were genetically identical with them except that they were homozygous wild-type for *keap1*. Data are shown as means \pm SE of three experiments performed in duplicate. (B) Male, but not female, heterozygous *keap1* flies show a significantly higher survival rate after exposure to Paraquat than their otherwise genetically identical wild-type sibling controls. Data are shown as means \pm SE of four experiments performed in triplicate. (C) Under standard culture conditions, male heterozygous *keap1* flies live significantly longer than their otherwise genetically identical wild-type sibling controls. Data are shown as percentage of flies alive at each age; 500–700 flies of each genotype and sex were assayed. This figure is adapted from Sykietis GP and Bohmann D, Keap1/Nrf2 signaling regulates oxidative stress tolerance and lifespan in *Drosophila*, *Dev Cell*, vol. 14, p. 76–85, Copyright 2008, with permission from Elsevier.

Parkinsonism [132, 133]. DJ-1 protects neurons from oxidative stress, but its functionality is lost upon oxidation [134]. Because DJ-1 stabilizes Nrf2 by preventing its association with Keap1, the oxidation of DJ-1 in PD could compromise Nrf2 signaling and precipitate the collapse of the antioxidant response system [135]. In summary, multiple independent lines of evidence highlight Nrf2 as an important factor that protects against PD.

In an effort to elucidate PD pathogenesis, several *Drosophila* models of the disease have been created by manipulating genes implicated in rare hereditary forms of early-onset parkinsonism. These include flies harboring loss-of-function mutations in the genes encoding parkin or transgenically overexpressing human α -synuclein in the DA neurons [136–138]. The resulting phenotypes are reminiscent of human PD and include premature loss of DA neurons, locomotor deficits, and

increased sensitivity to oxidative stress [136–143]. As genetic models of PD, these flies facilitate the in vivo discovery of genes, pathways, and compounds that may be useful for the prevention and/or treatment of this debilitating disorder. Loss-of-function *parkin* mutations lead to the degeneration of a cluster of DA neurons termed protocerebral posterior lateral 1 (PPL1). This neuronal loss was exacerbated by a loss-of-function allele of the detoxification gene *gstS1*; conversely, overexpression of *gstS1* specifically in DA neurons reduced *parkin*-associated DA neuronal loss [139]. Significant neuronal loss in the PPL1 cluster is also observed when human α -synuclein is expressed in DA neurons [138]. Loss-of-function mutations in *gstS1* or in *gclm*, which encodes the rate-limiting enzyme in the synthesis of the major antioxidant glutathione, further exacerbate the loss of DA neurons due to α -synuclein toxicity [138]. Conversely, overexpression of either of these enzymes

significantly protected DA neurons from α -synuclein-induced degeneration [138]. Importantly, the administration of the potent Nrf2 inducers allyl disulfide or sulphoraphane upregulated both *gstS1* and *gclm* and prevented the loss of DA neurons in both the parkin and α -synuclein fly models of PD [138].

More recently it was shown that decaffeinated coffee and nicotine-free tobacco, both of which can activate Nrf2 signaling, provide neuroprotection in the *parkin* and α -synuclein fly PD models [104]. RNAi-mediated knockdown of Cnc enhanced the toxicity of α -synuclein and suppressed the rescuing effect of decaffeinated coffee and nicotine-free tobacco [104]. Unfortunately, the transgene employed to knock down Cnc was non-isoform selective, that is, it was designed to target all three Cnc isoforms [104]. It therefore remains to be formally tested whether the Nrf2 homolog CncC is the isoform protecting DA neurons from α -synuclein toxicity. Moreover, it will be important to investigate whether the genetic activation of Nrf2 signaling is sufficient to ameliorate the toxicity of α -synuclein. A recent study in a *C. elegans* model of PD induced by exposure to the heavy metal manganese showed that SKN-1 activation is required to protect DA neurons from manganese-induced degeneration [144]. The species conservation of the protective role of Nrf2 against DA neuron degeneration supports the use of the genetically more tractable invertebrate model organisms for studies investigating Nrf2 as a preventive and/or therapeutic target in PD. In the near future, we anticipate that such studies will be expanded to other genetic models of human neurodegenerative diseases.

22.1.5.3 Nrf2 and Methylmercury Toxicity Methylmercury (MeHg) is an environmental pollutant with electrophilic chemical properties [145], to which humans can be exposed through diets rich in predatory fish like tuna and swordfish. MeHg easily crosses the blood-brain barrier and has potent neurotoxic properties [146]. Therefore, elucidating the mechanisms of resistance to MeHg is an issue of major public health importance. MeHg was found to interact with recombinant Keap1 in vitro, and Nrf2 overexpression could attenuate MeHg-induced cytotoxicity in cultured mammalian neuroblastoma cells [147]. Similarly, MeHg was shown to activate Nrf2 in cultured astrocytes and microglial cells, and inhibition of Nrf2 activation correlated with increased MeHg toxicity [148, 149]. In addition, primary hepatocytes from Nrf2-deficient mice accumulated more MeHg and were more susceptible to MeHg-induced cytotoxicity than control cells [147]. In contrast, primary hepatocytes from hepatocyte-specific conditional Keap1-deficient mice accumulated less MeHg and were more resistant to its toxic effect [147]. Thus the

activation of Nrf2 reduces MeHg toxicity, by both increasing its detoxification in the liver and augmenting the resistance of its target tissues.

Whereas a number of cytotoxic mechanisms of MeHg have been characterized in differentiated cells, its mode of action in the developing nervous system in vivo is less clear. In primate and rodent models, MeHg exposure has been shown to cause aberrant cell migration and disorganized patterning of the brain's cortical layers [150–152]. However, these animal models are not sufficiently accessible genetically to facilitate elucidation of the molecular and cellular pathways targeted by MeHg. Notably, a recent study established the *Drosophila* embryo as a platform for elucidating MeHg-sensitive pathways in neural development [153]. When developing fly embryos were exposed to MeHg, a dose-dependent inhibition of embryonic development was observed, evident as failure of the embryos to hatch to the larval stage. In addition, specific defects in neural development were documented, including abnormalities in neuronal and glial cell patterning consistent with disrupted migration; and pronounced defects in neurite outgrowth were observed in both central and peripheral neurons. Importantly, the ectopic expression of CncC enhanced embryonic development and hatching in the presence of MeHg [153]. Thus the protective role of Nrf2 against MeHg is conserved in *Drosophila*, highlighting the fly embryo as a facile model system for investigating mechanisms of MeHg resistance. Moreover, this work suggests that CncC may protect flies from a battery of heavy metals and environmental pollutants that they encounter in their natural environment. Consistently, in our initial characterization of CncC/Keap1 signaling we had shown that arsenic, another heavy metal and environmental pollutant known to activate Nrf2 [129], induces ARE-mediated transcriptional responses in *Drosophila* [88].

C. elegans has also been recently developed as a model for investigating the mechanisms of MeHg toxicity and resistance [154–156]. It was found that low, chronic exposure of worms to MeHg confers embryonic defects, developmental delays, decreases in brood size and animal viability, and, interestingly, DA neuron degeneration [156]. MeHg exposure resulted in the induction of glutathione-S-transferases [155, 156], which was largely dependent on SKN-1 [156]. Moreover, SKN-1 was shown to be expressed in the DA neurons, and reducing SKN-1 gene expression increased MeHg-induced animal vulnerability and DA neuron degeneration [156]. Taken together, these recent worm and fly studies highlight the utility of the genetically tractable invertebrate models for studies on MeHg toxicity and resistance. More such studies can be anticipated in the near future, including genetic screens for which these

organisms are ideally suited. The protective role of SKN-1 against MeHg toxicity to DA neurons [156] also prompts testing whether CncC has a similar role in flies, which would be consistent with the recent findings in the fly PD models reviewed in the previous section.

22.2 CONCLUSION

Because Nrf2 has emerged as a critical transcription factor for the maintenance of tissue homeostasis; the protection from oxidative and electrophilic stressors; and the prevention of diverse pathologies, it holds substantial promise as a therapeutic target for human diseases. To date, most progress in the Nrf2 field has resulted from studies in mice and cultured mammalian cells. Flies have recently been established as an invertebrate genetic model organism in which Nrf2 signaling is functionally conserved [88]. Thus, together with worms, they have the potential to complement vertebrates in this exciting research area. Through their unique experimental strengths, flies could also facilitate breakthroughs of potentially major impact on human health issues, such as the extension of the healthy life span, the prevention of neuronal degeneration, and the treatment of metabolic disorders [157], which are also generally accompanied by elevated levels of oxidative stress.

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ORCHESTRATION OF OXIDATIVE STRESS RESPONSES IN *DROSOPHILA MELANOGASTER*: A PROMOTER ANALYSIS STUDY OF CIRCADIAN REGULATORY MOTIFS

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23.1 INTRODUCTION

Circadian clocks are endogenous molecular regulators that coordinate daily changes in gene expression and cellular and physiological functions with external day/night cycles. The core circadian clock in the fruit fly *Drosophila melanogaster*, consists of interacting molecular feedback loops. In the main loop, transcriptional activators (called positive clock elements), encoded by genes *Clock* (*Clk*) and *cycle* (*cyc*), stimulate the expression of two other clock genes, *period* (*per*) and *timeless* (*tim*), in the early night [1]. This leads to periodic increases in the levels of *per/tim* mRNA and PER/TIM proteins. These proteins (called negative elements of the clock) accumulate in cell nuclei late at night and act as inhibitors of the CLK-CYC complexes, resulting in the suppression of *per* and *tim* transcription (Fig. 23.1) [2]. In the second feedback loop, daily oscillations of *Clk* mRNA are achieved via a rhythmically active transcriptional repressor, encoded by *vri* (*vri*), and transcriptional activator, encoded by *Pdp1ε* [3–6]. Rhythmic expression of *vri* and *Pdp1ε* is also activated by CLK-CYC complexes, via a similar mechanism as *per* and *tim* (Fig. 23.1).

CLK is a basic helix-loop-helix (bHLH) transcription factor that has a PER-ARNT-SIM (PAS) protein interaction domain. An approximately 69-base (b)

enhancer sequence is situated ~500 b upstream of the *per* transcription start site (TSS). Within this enhancer, a consensus “E-box” bHLH transcription factor binding site having the sequence CACGTG is required for transcriptional activation [7]. Likewise, a consensus CACGTG E-box, approximately 2.5 kilobases (kb) upstream of *tim*, has been known to be essential for its transcriptional activation [8, 9]. Recent studies have shown strong rhythmic binding of CLK to the E-box upstream of *per* [10, 11]. Also, there was weak rhythmic binding of CLK to the intronic E-box of *tim* [11].

The circadian rhythms generated by these clocks are responsible for 24-h oscillations in diverse biological processes. While the central genes governing circadian pacemaker rhythmicity have mostly been identified, clock-controlled output molecules responsible for regulating rhythmic behaviors remain largely unknown. Hence, identification of genes controlled by the central clock would be important in providing an avenue for understanding circadian oscillations in diverse biological processes including locomotor activity, feeding behavior, hormone secretion, digestion, and the onset of sleep. Genomewide studies of circadian gene expression revealed rhythms in the expression of multiple genes involved in various metabolic pathways and stress response in *D. melanogaster* [12–14]. A number of genes involved

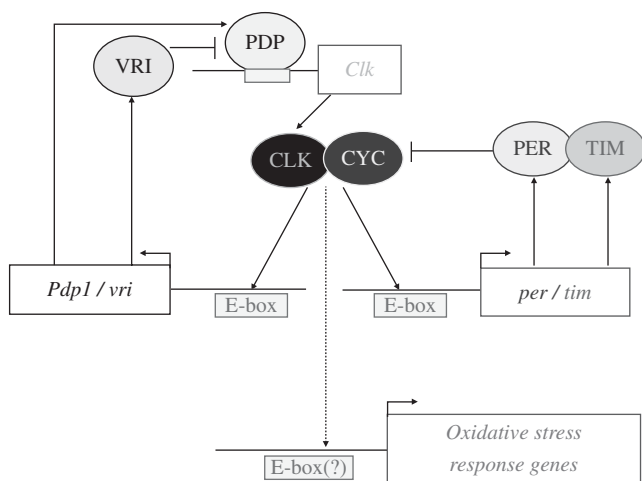


Fig. 23.1 Model of the *Drosophila* circadian clock. CLK and CYC heterodimers bind to E-boxes on *per* and *tim* promoters and activate their transcription during the day and early evening. PER and TIM proteins accumulate and translocate into the nucleus to repress their own activators CLK/CYC. During the day, PER and TIM are degraded, allowing a new cycle of transcription to start. In another loop, CLK/CYC activate transcription of *vri* and *Pdp1ε*, as VRI and PDP1ε proteins accumulate and translocate into the nucleus to inhibit and activate *Clk* transcription, respectively. We hypothesize that genes coding for various oxidative stress response genes could be under the control of the circadian clock (dotted arrow).

in oxidative stress responses were found to be expressed in a daily rhythm. We have previously reported that there is a circadian regulation in the response to oxidative stress [15], suggesting a concerted circadian expression in the antioxidant or cytoprotective genes in response to reactive oxygen species (ROS) or redox stress. We also reported a significant accumulation of oxidative damage to proteins (protein carbonyls) in *period* null (*per⁰¹*) mutants compared to wild-type flies [15, 16], similar to a previous study [17]. Increase in the susceptibility of clock-disrupted flies to exogenous oxidative stressor would imply that these flies have either impaired antioxidant and/or reduced repair systems. However, in flies with a functional clock, these systems are apparently unaffected [15, 16].

Against this backdrop, we hypothesize that the genes coding for different oxidative stress responses may be regulated by the circadian clock directly or indirectly. If this hypothesis is true, such genes should have binding sites for clock elements in their promoter regions, upstream of the TSS. We conducted a bioinformatics-based promoter analysis of major oxidative stress responsive systems in *Drosophila* with two main objectives: (i) to identify the putative conserved circadian regulatory sequence (E-box) and (ii) to identify novel conserved motifs in the promoter regions and predict their function with online bioinformatics tools (Fig. 23.2).

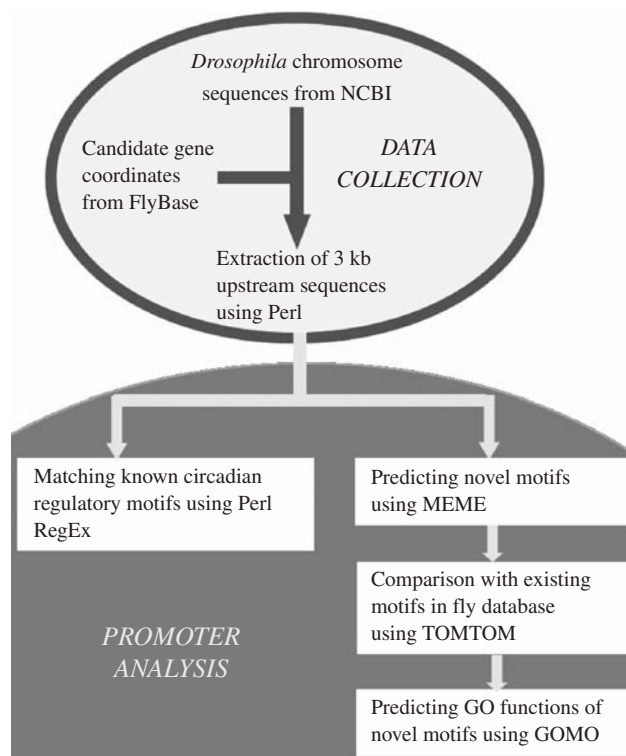


Fig. 23.2 Sequential procedure of the study. Data collection consisted of obtaining *Drosophila* chromosome sequences and coordinates of candidate genes and extraction of 3-kb upstream sequences. Promoter analysis comprised a search for known circadian regulatory motifs and prediction of novel putative motifs with online bioinformatics tools.

23.2 PROMOTOR ANALYSIS

23.2.1 Chromosome Sequences and Gene Coordinates

The *D. melanogaster* chromosome sequences (NT 004354, NT 033777-79, NT 037436) were downloaded from the National Center for Biotechnology Information (NCBI) genome database ([http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&term=Drosophila%20melanogaster\[orgn\]](http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genome&term=Drosophila%20melanogaster[orgn])). The coordinates and strand information for every candidate gene were obtained from FlyBase (<http://flybase.org/>) (Table 23.1).

23.2.2 Extraction of Promoter Sequences

Two-kilobase upstream sequences for each gene coding region were extracted with the substring function of Perl, a high-level, general-purpose UNIX scripting interpreted programming language.

23.2.3 Verification of Promoter Regions

The promoter sequences were subjected to BLASTn in FlyBase (<http://flybase.org/>) to verify their correct locations.

TABLE 23.1 Location of consensus E-box (CACGTG) sequences upstream of the transcription start site (TSS) in the candidate genes*

Gene ID	Gene Name	Chromosome: Coordinates (Strand)	E-Box Location
CG2647	<i>period (per)</i>	X: 2579613.2586813 (+)	525, 1399, 1477
CG17888	<i>Par domain protein 1 (Pdp1)</i>	3L: 7807437.7860472 (+)	1848
CG3962	<i>Kelch-like ECH-associated protein 1 (Keap1)</i>	3R: 12899661.12905665 (–)	1735
CG17894	<i>cap-n-collar (cnc)</i>	3R: 19011300.19047683 (–)	1618
CG11793	<i>Cu/Zn Superoxide dismutase (Sod)</i>	3L: 11105381.11106838 (–)	
CG8905	<i>Mn Superoxide dismutase 2 (Sod2)</i>	2R: 12660421.12661413 (–)	
CG6871	<i>Catalase (Cat)</i>	3L: 18815706.18821294 (+)	
CG2151	<i>Thioredoxin reductase 1 (Trxr1)</i>	X: 8136517.8142072 (+)	
CG11401	<i>Thioredoxin reductase 2 (Trxr2)</i>	3L: 22543326.22545122 (+)	
CG31884	<i>Thioredoxin 2 (Trx2)</i>	2L: 9613165.9616048 (+)	
CG3315	<i>Thioredoxin-T (TrxT)</i>	X: 5204614.5205921 (–)	
CG10964	<i>sniffer (sni)</i>	X: 8135531.8137337 (–)	
CG6835	<i>Glutathione Synthetase (GS)</i>	X: 17783580.17792636 (+)	901
CG2259	<i>Glutamate-cysteine ligase catalytic subunit (Gclc)</i>	X: 7996613.8010177 (–)	89, 1436
CG4919	<i>Glutamate-cysteine ligase modifier subunit (Gclm)</i>	3R: 18511022.18512249 (–)	2749
CG10045	<i>Glutathione S transferase D1 (GstD1)</i>	3R: 8193269.8194987 (–)	
CG5164	<i>Glutathione S transferase E1 (GstE1)</i>	2R: 14285898.14286728 (+)	
CG8938	<i>Glutathione S-transferase S1 (GstS1)</i>	2R: 12980758.12984935 (–)	
CG7266	<i>Ecdysone-induced protein Eip71CD (msrA)</i>	3L: 15504153.15506302 (+)	2544
CG6584	<i>SelR (msrB)</i>	3R: 6689071.6694379 (–)	

*The *period* and the *Pdp1* gene were used as controls to verify the Perl RegEx script.

23.2.4 Matching E-Box Elements with Perl Regular Expression

The presence and location of the consensus circadian regulatory sequence (E-box) were detected with Perl Regular Expression (RegEx).

23.2.5 Identification of Novel Conserved Motifs and Comparison with the Fly Database

Novel conserved motifs (6–10 bases) in the promoter regions were identified with the motif discovery tool Multiple Elm for Motif Elicitation (MEME) http://meme.sdsc.edu/meme4_3_0/intro.html [18]. Putative novel motifs were compared to a database of fruit fly motifs, FLYREG v2, with the motif comparison tool TOMTOM. The Sandelin–Wasserman similarity function was chosen for motif column comparison with a significance threshold q value of 0.5. The q value is the estimated false discovery rate if the occurrence is accepted as significant [19].

23.2.6 Predicting Functions of Novel Motifs

Gene Ontology (GO) terms and functions associated with the identified novel motifs in the promoter regions were searched with the software Gene Ontology for Motifs (GOMO v4.3.0) http://meme.sdsc.edu/meme4_3_0/cgi-bin/gomo.cgi [20].

23.3 RESULTS

23.3.1 Characterization of Circadian Regulatory Sequences

The consensus E-box sequence (CACGTG) was found in 6 of the 18 candidate genes. Three consensus E-box elements were identified in the *per* promoter 525, 1399, and 1477 bases upstream of the TSS (Table 23.1, Fig. 23.3). The E-box element in *per* has been previously characterized and identified by functional studies [7, 10, 11, 21]; thus this gene served as our control to test the successful functioning of the Perl RegEx. A single E-box was identified in the promoter regions of *Pdp1*, *Kelch-like ECH-associated protein 1 (Keap1)*, *cap-n-collar (cnc)*, *Glutamate cysteine ligase modulatory (Gclm)* subunit, and *methionine sulfoxide reductase (msrA)* genes, whereas two E-boxes were found in *Glutamate cysteine ligase catalytic (Gclc)* subunit gene.

23.3.2 Identification of Novel Conserved Motifs

The promoter sequences of candidate genes were further subjected to predictive analysis using MEME for searching potential novel putative motifs that were common to them. Four such motifs, 6–10 bases long with a log likelihood ratio of 185 and highly significant expect (e) value, were identified (Fig. 23.4). Furthermore, these novel motifs were subjected to a comparison with the

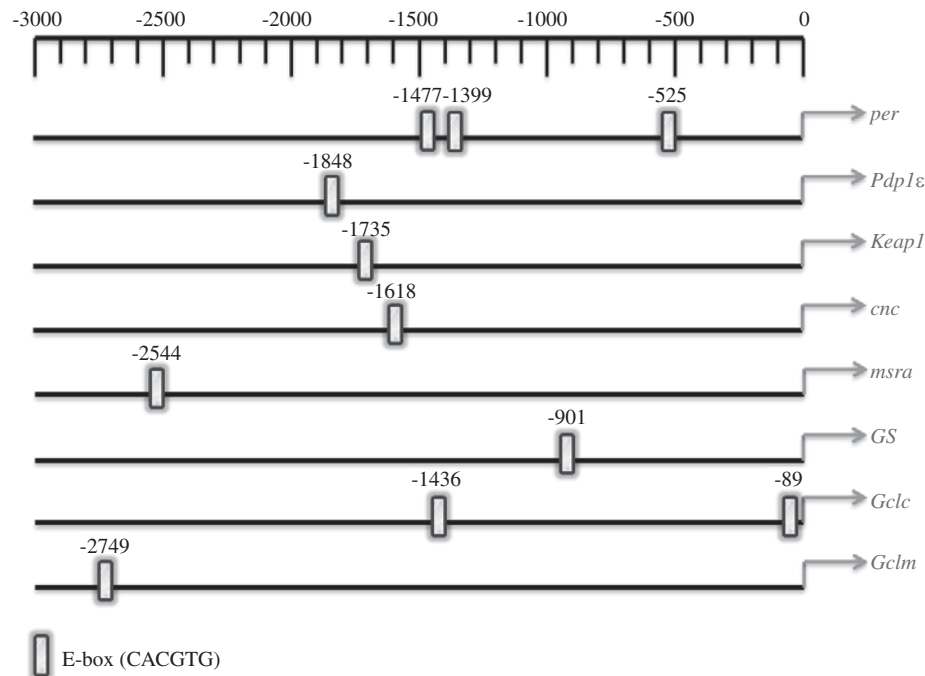


Fig. 23.3 Block diagram showing the relative location of consensus circadian regulatory sequences (colored boxes) upstream of the transcription start site (green arrows) in different candidate genes. The canonical E-box, 2822 bases upstream of *tim*, has not been depicted in this figure.

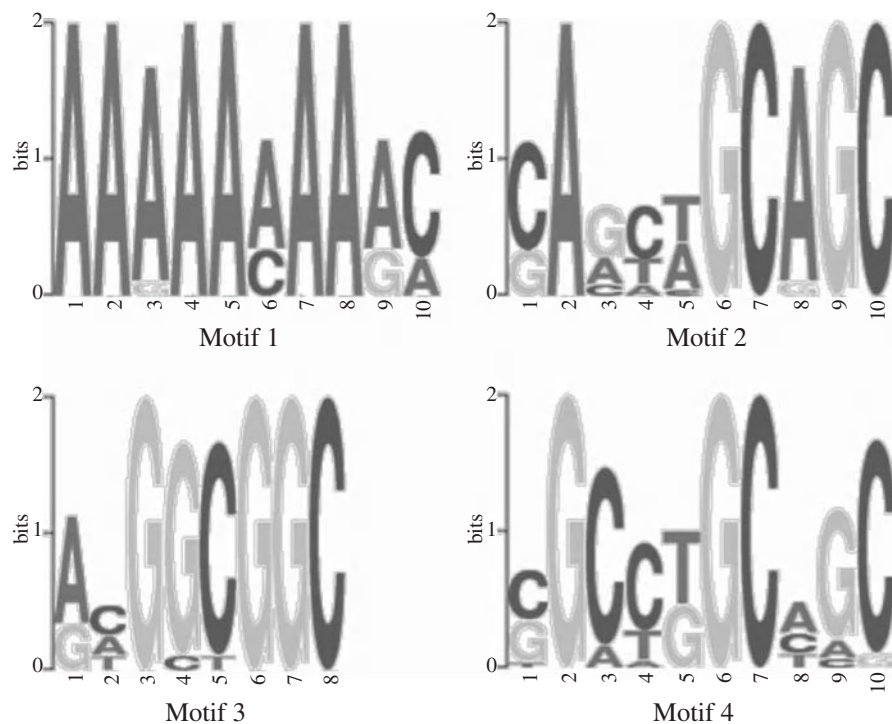


Fig. 23.4 Sequence LOGOS of the MEME motifs 1–4 displaying the probability of each base appearing at every possible position in the motif. The total height of the stack is the information content of that position in the motif in bits. The height of the individual letters in a stack is the probability of the letter at that position multiplied by the total information content of the stack. (See color insert.)

existing motifs in the fruit fly motif database FLYREG database-Bergman and Pollard v2 with TOMTOM. The motifs were compared by using the Sandelin–Wasserman similarity function, and only statistically significant matches ($P < 0.001$) with a low false discovery rate (q value) less than 50% were obtained.

Three of the four novel motifs had significant alignments with existing motifs in the database. Motif 1 aligned with the existing *hb* motif (P value = 0.00012, q value = 0.018) (Fig. 23.5A). No significant match was obtained for motif 2. Motif 3 produced significant alignments with three existing motifs in the database, *Med* (P value = 0.0038, q value = 0.021), *Mad* (P value = 0.0038, q value = 0.021), and *brk* (P value = 0.0096, q value = 0.035) (Fig. 23.5B). Motif 4 aligned to the *Med* (P value = 0.0014, q value = 0.041) and *Mad* (P value = 0.0021, q value = 0.3) motifs (Fig. 23.5C).

23.3.3 Predicting Functions of Novel Motifs

GO terms and functions associated with the novel motifs in the promoter regions were searched with GOMO v4.3.0. Interestingly, motif 4 was predicted to be a transcription factor binding site for the regulation of transcription, locomotor rhythms, and the entrainment of the circadian clock (Table 23.2). Further, motif 2 was identified to be a potential site for the binding of casein kinase 2 (CK2), a serine/threonine protein kinase that is known to phosphorylate clock proteins, resulting in their circadian rhythmicity [22, 23]. Motifs 1 and 3 were predicted to have general housekeeping functions like triplet codon-amino acid adaptor activity and regulation of biosynthetic processes, respectively (Table 23.2).

23.4 DISCUSSION

The consensus E-box bHLH transcription factor binding site has been shown to mediate rhythmic expression of several core clock genes [7, 11]. The presence of this consensus sequence in the promoter regions of six antioxidant genes in *Drosophila* suggests likely circadian control in their regulation.

Keap1 and *cnc* both revealed the presence of the typical E-box in their promoters. Members of the CNC (cap ‘n’ collar)-basic leucine zipper family of transcription factors are principal mediators of defensive responses to redox stress [24]. The *cncC* isoform transcribed off the *Drosophila cnc* locus has been suggested to be the counterpart of mammalian *Nrf2* (NF-E2-related factor 2), which contains the KEAP1 binding ETGE motif and an upstream hydrophobic region [25, 26]. The *Drosophila* genome also harbors a homolog of vertebrate *Keap1* genes. This gene (CG3962) and its protein

product (dKeap1) have been shown to have striking similarity with mammalian and zebrafish homologs. The physical association between dKeap1 and CncC has previously been suggested by a genomewide yeast two-hybrid (Y2H) experiment [27]. This interaction predicts that dKeap1 should act as a negative regulator of CncC in vivo. It has been demonstrated that dKeap1/CncC signaling regulates oxidative stress responses in *Drosophila* [26] and could also function in xenobiotic stress responses. Circadian expression profiling of drug-processing genes and transcription factors in mouse has revealed that both *Keap1* and *Nrf2* are rhythmic with a 50% increase in *Nrf2* expression at 2 P.M., whereas the expression of its cytoplasmic repressor *Keap1* was shown to be higher (40%) during the dark phase than during the light phase [28]. A similar rhythmic pattern of these genes remains to be identified in the case of *Drosophila* because of circadian binding elements in their promoters.

While diurnal rhythms in the activity of various antioxidant enzymes have been described from various phylogenetically distant organisms [29], not many studies have actually been able to demonstrate rhythms at the mRNA level. In this study, the canonical E-box was not detected in any of the classical antioxidant enzyme genes. The circadian clock has been reported to gate the expression of two *Catalase* genes (*CAT2* and *CAT3*) in *Arabidopsis* to distinct opposite circadian phases [30]. Rhythms in catalase activity has been reported in mouse brain, kidney, and liver [31], while epigenetic inactivation of circadian clock gene *BMAL1* in hematologic malignancies in human cell lines has been reported to disrupt circadian expression pattern of *catalase* [32]. However, we have not discerned any circadian expression pattern in *Cat* gene expression in *Drosophila* [15]. Neither *Cu/Zn Superoxide dismutase (Sod)* nor *Mn Superoxide dismutase (Sod2)* revealed any of the putative circadian binding elements in their promoter regions.

Sniffer protein (encoded by *sni*) is an NADPH-dependent carbonyl reductase belonging to the enzyme family of short-chain dehydrogenases/reductases (SDRs). *sni* has been directly implicated in the cellular defense mechanism against oxidative stress in *Drosophila* [33], and not much is known about its circadian regulation. *Trxr1* has been reported to substitute for the glutathione reductase system in *Drosophila* and plays a crucial role in the thiol-based glutathione recycling system [34], which is essential to combat oxidative stress. Similarly, *Trx2*, *msrA*, *msrB*, and *GstS1* have also been reported to participate in oxidative stress responses in *Drosophila* [35–37]. While some of these genes participate directly in elaborating an antioxidant defense response, there are others such as methionine sulfoxide reductases (*msr*) that perform a role in repair of oxidatively damaged proteins.

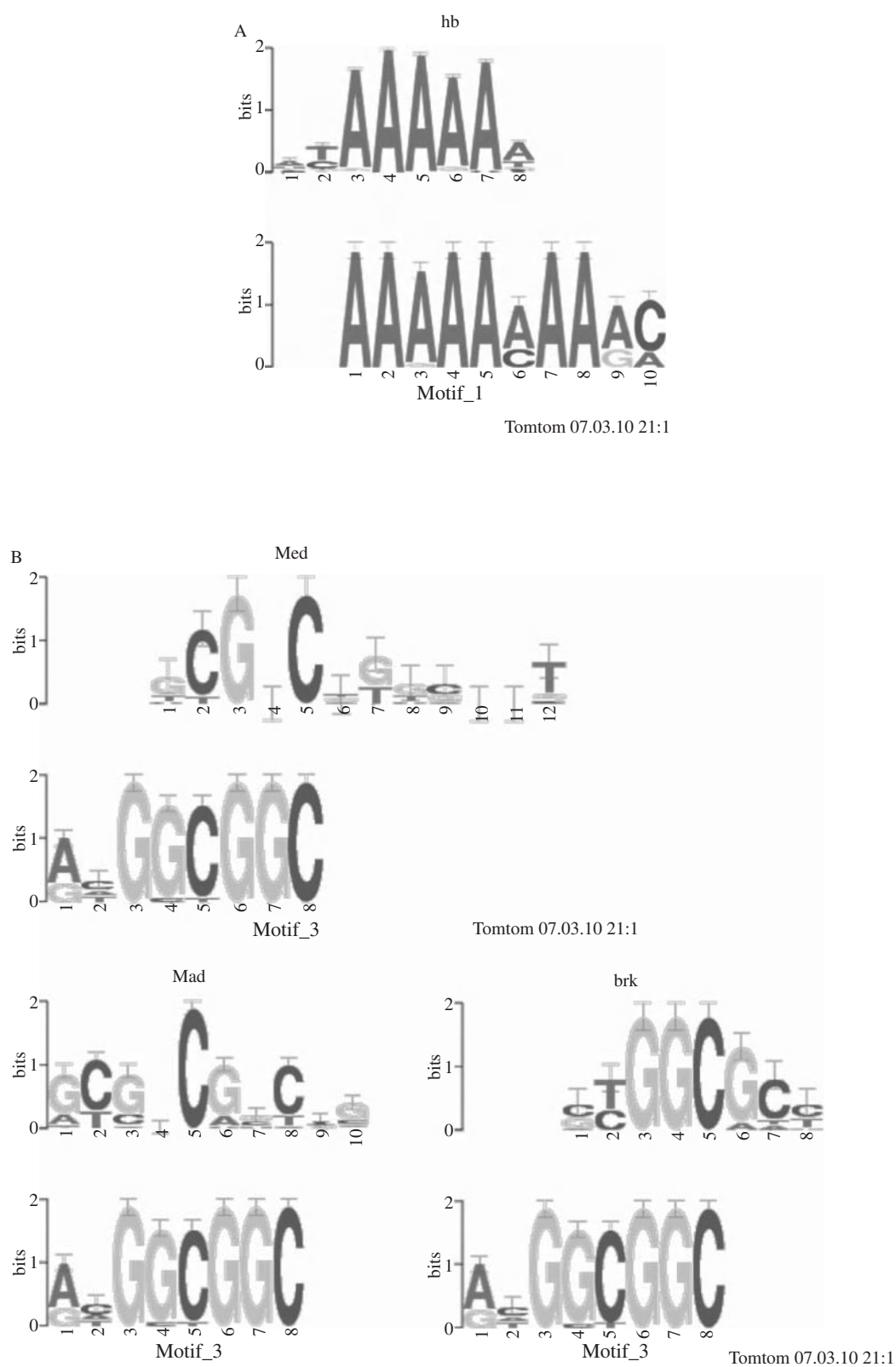


Fig. 23.5 TOMTOM output of the comparison of MEME motifs with existing motifs in the *Drosophila* database (FLYREG; Bergman and Pollard v2). Only statistically significant matches are displayed ($P < 0.001$) with a low false discovery rate ($q < 0.5$). (A) Motif 1. (B) Motif 3. (C) Motif 4. (See color insert.)

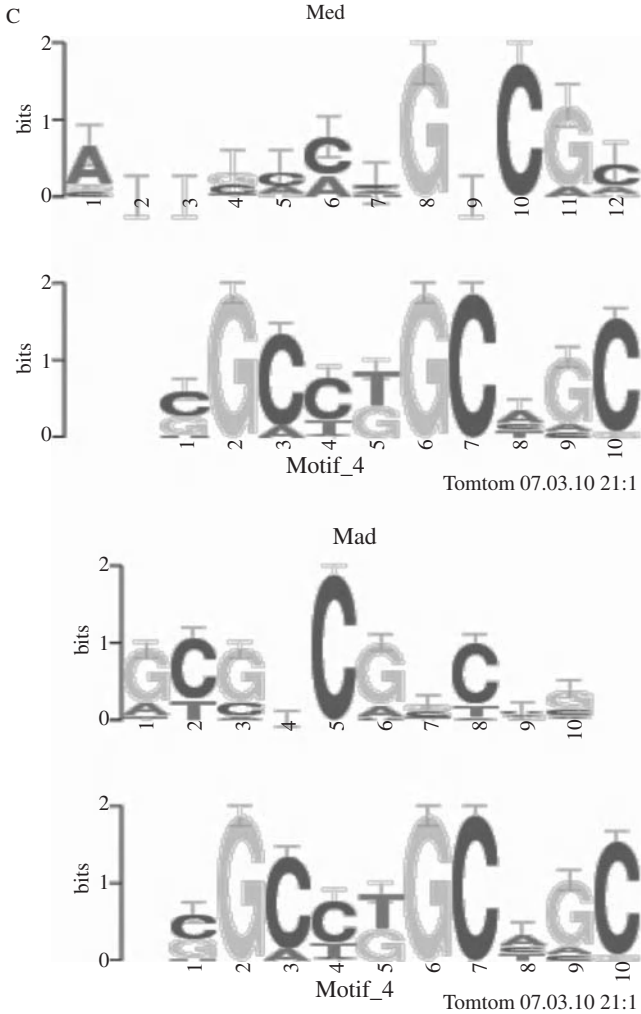


Fig. 23.5 (Continued)

Additionally, the tripeptide L-γ-glutamyl-L-cysteinylglycine, that is, glutathione (GSH), is the most abundant cellular non-protein thiol and has multiple physiological roles in vivo, acting as a cellular redox buffer, a potent nucleophile, and an antioxidant against ROS, and recent

evidence also suggests that intracellular GSH is a key regulator of stress-activated signal transduction pathways [38]. GSH is synthesized by glutamylcysteine ligase (GCL), which is the primary rate-limiting reaction in the pathway. GCL is heterodimeric, having a catalytic subunit (*Gclc*) and the modulatory subunit (*Gclm*). Both of these genes showed the canonical E-box consensus sequence.

The E-box is a widely used DNA control element. It affects many different genetic programs, including proliferation, differentiation, tissue specific responses, and cell death. The circadian clock is also one among many pathways that employ the E-box for establishing the robust waves of gene expression characteristic of circadian transcription. However, presence or absence of E-box alone in the promoter regions of genes may not be sufficient to guarantee control by the clock. The regulatory flexibility of the E-box hinges on the sequence ambiguity allowed at its core, the strong influence of the surrounding sequences, and the recruitment of spatially and temporally regulated E-box binding factors [39]. However, lack of an E-box in the promoter region of a gene does not necessarily mean that the gene may not be under circadian control. Hence, other motifs that are conserved in the promoter regions of genes under likely circadian control may serve as binding sites for clock transcription factors (TFs).

TF binding sites in the promoters of clock genes and oxidative stress responsive genes used in this study revealed novel conserved motifs that had significant matches with existing *Drosophila* motifs in the database (Fig. 23.4, Fig. 23.5). One of these motifs (motif 4) had a predicted functional role in the entrainment of circadian rhythms and locomotor function, while another (motif 2) was a predicted binding site for casein kinase 2 (CK2), known to phosphorylate clock proteins [23, 40], playing an important role in their stability and function (Table 23.2). However, the presence of a variety of circadian elements that suggest control of promoter activity is not the only manner of control of rhythmic activity. In

TABLE 23.2 Gene Ontology (GO) functions associated with the novel MEME motifs in the promoter regions of candidate genes using GOMO v4.3.0

Motif	GOMO Score	P Value	Q Value	Accession No.	GO Definition
1	2.09e-10	1.42e-06	1.73e-03	GO:0000499	<i>molecular_function</i> : base pairing with mRNA
	2.09e-10	1.42e-06	1.73e-03	GO:0030533	<i>molecular_function</i> : triplet codon-amino acid adaptor activity
2	2.39e-05	1.42e-06	9.44e-03	GO:0005956	<i>cellular_component</i> : protein kinase CK2 complex
3	5.16e-04	1.37e-04	2.57e-02	GO:0034984	<i>biological_process</i> : cellular response to DNA damage stimulus
4	5.26e-04	1.42e-04	2.57e-02	GO:0009889	<i>biological_process</i> : regulation of biosynthetic process
	9.82e-05	3.13e-05	4.47e-02	GO:0045449	<i>biological_process</i> : regulation of transcription
	2.02e-04	5.55e-05	4.47e-02	GO:0045475	<i>biological_process</i> : locomotor rhythm
	3.58e-04	1.10e-04	4.70e-02	GO:0009649	<i>biological_process</i> : entrainment of circadian clock

some instances, nonrhythmic mRNA expression can result in rhythmic protein expression; for example, in the dinoflagellate *Gonyaulax polyedra*, luciferin binding protein (LBP) mRNA is not rhythmic, but protein expression (and bioluminescence) exhibits clear circadian oscillation mediated by the rhythmic binding activity of the RNA binding protein CCTR to the *LBP* 3' UTR [41]. Similarly, rhythmic expression of the deadenylase nocturnin in *Xenopus* retina may potentially regulate steady-state downstream target mRNAs to generate protein cycles [42, 43]. Circadian oscillations at the protein level but not in the mRNA level have also been reported in mouse liver [44]. Thus, although the present analysis indicates that different antioxidant systems may have binding sites for circadian clock TFs and may be directly or indirectly controlled by the circadian clock, it is still largely predictive in nature. Further functional studies are needed to substantiate the results obtained in this study. This may include chromatin immunoprecipitation (ChIP) to find potential TFs binding to the novel motifs and the use of Y2H screens with reporter genes.

23.5 SUMMARY AND CONCLUSION

The regulation of gene expression (specifically in response to oxidative stress) by the circadian clock has emerged as a novel subdiscipline in molecular biology and has promising therapeutic implications. While the identification of genes controlled by the central clock would be important to understand the diverse biological processes under circadian control, most studies have stagnated at the level of microarray data. Our study moves a step beyond and identifies candidate genes likely to be under circadian transcriptional control. While these studies are no doubt important, further characterization of the genes would be required for a greater understanding of how the clock orchestrates a concerted protective function in an organism in response to oxidative stress. Thus a more detailed investigation is to be undertaken to validate their control by the circadian clock. Many of these genes are not only likely to prove integral to the fly but could also lead to the identification of homologous genes in mammalian systems.

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THE PROTECTIVE ROLE OF SESTRINS AGAINST CHRONIC TOR ACTIVATION AND OXIDATIVE STRESS

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24.1 INTRODUCTION

Oxidative stress is an undesirable consequence of oxidative respiration, which our cells utilize to produce the required levels of energy for life processes. Reactive oxygen species (ROS), a side product of mitochondrial respiration, can damage many important macromolecules, including DNA, lipids, and proteins. Therefore, complex mechanisms have evolved to defend cellular architecture and genomic information against damage produced by ROS. One well-characterized defense mechanism is performed by redox regulating enzymes, including catalase, peroxidase, and superoxide dismutase, which can directly scavenge or eliminate ROS [1]. Another defense mechanism is the stress-induced signal transduction pathway, which can block cell growth and promote repair of damaged macromolecules such as DNA under conditions of oxidative stress [2, 3]. During oxidative stress, ROS-induced damage of mitochondrial DNA, lipids, and proteins can decrease respiration efficiency, provoking mitochondria to compensate and produce more ROS. To avoid this destructive cycle, cells eliminate their damaged mitochondria through a mechanism called mitophagy—mitochondria-specific autophagy [4, 5].

In this chapter, we discuss a unique family of proteins, the Sestrins, that is critically involved in the cellular defense system against ROS (Fig. 24.1) [6, 7]. Sestrins are induced by oxidative stress-induced

signaling pathways, and they protect cells and organisms from the detrimental consequences from oxidative stress by several means [8]. One activity of Sestrins is promotion of recycling of peroxiredoxins, which are important ROS-scavenging small proteins [1]. This effect of Sestrins contributes to elimination of ROS during oxidative stress [9]. A separate activity of Sestrins is inhibition of signaling mediated by the target of rapamycin complex 1 (TORC1). This activity of Sestrins can stop cell growth and funnel the saved energy expenditure into macromolecular repair machinery [10]. In addition, by inducing autophagy that eliminates damaged mitochondria, Sestrins contribute to mitochondrial quality control [11]. Sestrin deficiency therefore leads to cellular ROS accumulation, which can cause diverse ROS-associated pathologies such as muscle degeneration and cardiac dysfunction [12].

24.2 SESTRIN—A UNIQUE GENE FAMILY

The first discovered Sestrin family protein was Sestrin 1 (Sesn1), which was identified as a p53 target gene in a differential display screen using a cell line expressing a temperature-sensitive mutant of p53 [13] and was originally named PA26 (p53-activated gene 26). The *cis*-regulatory sequences in the *Sesn1* genomic locus contain p53 binding sites, and *Sesn1* is induced by virtually every p53-activating stimulus. However, the function of

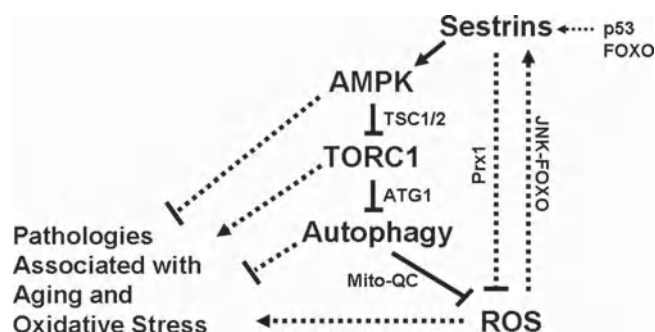


Fig. 24.1 Role of Sestrins against oxidative stress and aging. Sestrin has two independent biochemical roles against oxidative stress: (i) Sestrins can reduce ROS by promoting peroxiredoxin recycling, and (ii) Sestrins inhibit chronic TORC1 that suppresses autophagy, which is critical for eliminating ROS-producing dysfunctional mitochondria. Through these activities Sestrins can prevent diverse age-associated pathologies such as fat accumulation and cardiac and skeletal muscle degeneration.

Sesn1 remained elusive because it does not contain any known domains or motifs that can be used to infer its molecular functions. Three years later, the second member of the Sestrin family, Sestrin 2 (Sesn2), was isolated in a microarray analysis designed to identify novel hypoxia-inducible genes [14] and, consequently, was originally named Hi95 (hypoxia-inducible gene 95). Sesn2 shares a high degree of amino acid sequence similarity with Sesn1. Finally, bioinformatic analyses identified the third member of Sestrin family, Sesn3, which is also closely related to the other two Sestrins. Sesn1, Sesn2, and Sesn3 constitute a unique protein family that does not share any obvious sequence homologies to other proteins.

Sestrins are found throughout the animal kingdom, as well as some protistan species [6]. Sestrin genes are found in a single copy in most invertebrate protostome species as well as hemichordates of the deuterostome lineage. However, in the vertebrate lineage, the Sestrin locus was triplicated and diverged into Sesn1, Sesn2, and Sesn3 (Table 24.1). Interestingly, similar triplication and divergence were also found for the p53 gene [15]. However, there are no currently known immediate homologs of Sestrin or p53 in the genome of yeast, plant, or bacteria, suggesting that these genes arose selectively during evolution of the animal kingdom.

24.3 REGULATION OF SESTRIN EXPRESSION BY STRESSES

24.3.1 Genotoxic Damage

As p53 transcriptional targets, Sesn1 and Sesn2 can be activated by most of the genotoxic stresses that induce

TABLE 24.1 Conservation of genetic components of Sestrin-related signaling pathways. Currently known homologs of Sestrin, p53, and catalytic subunits of AMPK, TOR, and ATG1 in yeast (*S. cerevisiae*), worm (*C. elegans*), fly (*D. melanogaster*), and mouse (*M. musculus*) are listed

<i>S. cerevisiae</i>	<i>C. elegans</i>	<i>D. melanogaster</i>	<i>M. musculus</i>
	cSesn	dSesn	Sesn1 Sesn2 Sesn3
	Cep53	dp53	p53 p63 p73
SNF1	AAK-1 AAK-2	dAMPK	AMPK α 1 AMPK α 2
Tor1 Tor2	CeTOR	dTOR	mTOR
ATG1	Unc-51	dATG1	ULK1 ULK2

p53 expression, including doxorubicin and UV and gamma-irradiation [13, 14]. The *Sesn1* genomic locus contains functional p53 binding sites, which may mediate the p53 inducibility of Sesn1 [13], although functional p53 sites in *Sesn2* locus have yet to be identified. Sesn1/2 induction after genotoxic damage requires p53, since p53-knockout cells have defects in the Sesn1/2 induction [13, 14]. Genomic instability caused by deficiency of the mitotic regulator Securin can also cause Sesn1 upregulation [16].

24.3.2 Hypoxia

Sesn1 and Sesn2 are inducible upon hypoxic insult, which is accomplished in a p53-independent fashion [14]. It has been proposed that hypoxia-inducible factor 1 (HIF-1) may be involved in the regulation of Sesn1/2 [17]. Cells in the mouse brain strongly expresses Sesn2 in response to hypoxia induced in an experimental model of acute stroke [14], suggesting that Sesn2 may have a neuroprotective role against ischemic injury. In macrophages, Sesn2 was also among the top 14 genes significantly induced by hypoxia and nitric oxide [18]. The induction of Sesn2 in macrophages is believed to be important in the regulation of peroxide signaling [17].

24.3.3 Oxidative Stress

Oxidative stress by H₂O₂ treatment also induced Sesn1/2 in cultured cell lines [9, 14]. The induction of Sesn2 upon oxidative stress is p53 independent in most cell lines [9, 14], although in some cell lines it is dependent on p53 and its downstream target p53-induced nuclear protein 1 (Inp1) [19]. The FoxO signaling pathway, which can be

activated by oxidative stress [20], may mediate the induction of Sesn1/2/3 during oxidative stress [20, 21].

24.3.4 Developmental and Environmental Ques

In *Xenopus*, Sesn1 was reported to be developmentally expressed in the notochord [22], while in mice, Sesn1 is mostly expressed in skeletal muscle [13, 23]. *Drosophila* Sestrin is also enriched in skeletal muscle (indirect flight muscle) [12]. In *Drosophila*, dSesn expression increases upon maturation and aging [12]. In short-lived mice recuperated from maternal protein restriction, Sesn1 expression in kidney was significantly downregulated [24]. In mouse lung, restraint in nose-only exposure tubes by itself can induce expression of Sesn1, as well as other stress-inducible genes [25]. In humans, Sesn1 is upregulated in peripheral blood mononuclear cells of chronic fatigue syndrome patients [26].

24.3.5 Chronic TORC1 Activation

In *Drosophila* cells, genotoxic damage-, hypoxia- or oxidative stress-dependent induction of *Drosophila* Sestrin (dSesn) were not observed, in contrast to mammalian cells, although gamma-irradiation-dependent induction of dSesn can be observable in first instar larvae [8]. Nevertheless, dSesn did accumulate in tissues in response to chronic TORC1 activation, which is associated with elevation of oxidative stress [12]. Accumulation of ROS and activation of JNK-FoxO signaling pathways are both required for this induction of dSesn [12]. In mice, Sesn1 expression is significantly increased in PTEN-deleted prostate cancer tissue relative to normal prostate tissue [27]. However, in human fibroblasts, oncogenic activation of Ras or AKT causes downregulation of Sesn1/3, which results in oxidative senescence [21, 28].

24.3.6 Regulation of Sestrins in Nervous System

It has been shown that Sesn1/2 are induced upon the activation of NMDA receptor in neurons [29] through increased histone acetylation [30]. Treatment of neuronal cells with neurotoxin amyloid- β significantly elevated the level of Sesn2 [31]. HIV-1 infection of mouse brains through nasal spray also caused upregulation of Sesn2 [32]. The induced Sestrins may protect neurons against oxidative, proteotoxic, and virus-induced damage [33].

24.3.7 Chemical Induction of Sestrins

Induction of Nur77, an orphan nuclear receptor, by methylene-substituted diindolylmethanes was known to induce Sesn2 [34], and Rosiglitazone, an antidiabetic drug, can induce Sesn1 in retinal cells [35]. Pyrrolidine

dithiocarbamate, which can cause oxidative stress in vivo, induces Sesn2 in human fibroblasts [36].

24.4 SESTRIN AS A REDOX REGULATOR

Peroxiredoxins (Prx) are evolutionarily conserved peroxidases found from bacteria to mammals that are major scavengers of endogenously produced ROS [37]. Overoxidation of Prx inactivates its redox activity, which requires reactivation by another oxidoreductase [38]. The bacterial AhpD protein is an oxidoreductase that can regenerate Prx [39] and has distant sequence homology to Sestrins [9], suggesting that Sestrins may have an oxidoreductase function that reduces ROS. Indeed, silencing of Sesn1 or Sesn2 increased cellular oxidative level in both basal and H₂O₂-treated conditions, while overexpression of Sesn1 or Sesn2 can decrease intracellular ROS [9]. Cys130 of Sesn1 and Cys125 of Sesn2 are equivalent to critical redox-active cysteine residues in the AhpD protein. Thus mutation of the cysteine residues can abolish the ROS-reducing activity of Sesn1 and Sesn2 [9]. Sesn1/2 can physically interact with Prx inside the cells and can change the redox state of Prx [9]. However, recombinant Sesn2 does not function as a oxidoreductase for Prx in vitro [40], suggesting that Sesn2-dependent regeneration of Prx may be indirect.

As Sesn1/2 are targets of p53, Sestrins' redox activity contributes to antioxidant function of p53, which is important for its tumor-suppressing activity against certain cancers such as lymphoma [41]. Mice expressing single extra copies of p53 and ARF, which have higher p53 activity, are characterized by cancer resistance, delayed aging, and decreased oxidative damage [42]. These phenotypes are associated with dramatic upregulation of Sesn1/2 [42], which can reduce cellular oxidative stress. Conversely, *p53^{Ser15Ala}* hypomorphic mutant mice, which exhibit reduced levels of Sesn1/2/3 expression, display metabolic derangements associated with redox deregulation [43]. Therefore, Sesn1/2 contribute to protecting p53-activated cells against oxidative insults, in combination with other antioxidant targets of p53 such as glutathione peroxidase 1, superoxide dismutases, and catalase [44, 45].

Sestrins' antioxidant function is observed in diverse cellular and physiological contexts. In cancer cell lines, Sesn1/2 protect cells from oxidative damage-induced cell death [9, 14]. Sesn3 is also important in reducing oxidative stress, and FoxO-induced Sesn3 expression can suppress premature oncogenic senescence caused by ROS accumulation [21]. In macrophages, Sesn2 plays an important role in peroxide defense [17]. In neurons, Sesn1/2 is induced upon synaptic activity and reduces

neuronal oxidative damage [29, 33]. Since *Sesn2* is induced upon ischemic insults in rat brain [14], *Sesn2* may also have neuroprotective functions against ischemia-reperfusion injury that induces mitochondrial oxidative burst [8].

24.5 OVERVIEW OF TORC1 SIGNALING

Another important output of Sestrin is target of rapamycin (TOR), which is a critical regulator of cell growth, development, and physiology [46–51]. TOR is a large protein kinase (289 kDa) and participates in two distinct protein complexes, TORC1 and TORC2 [51]. Among the TOR complexes, rapamycin-sensitive TORC1, composed of TOR, Raptor, PRAS40, and G β L, is the nutrient-sensing TOR complex. TORC1 can stimulate cellular anabolism of proteins and lipids while inhibiting autophagy (Fig. 24.2) [51]. TORC1 stimulates protein translation by phosphorylating p70 ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4E-BP) [47]. TORC1 facilitates lipid synthesis through lipogenic transcription factor sterol-responsive element binding protein (SREBP) [52]. In addition, TORC1 phosphorylates and inhibits an autophagy-initiating protein kinase complex composed of ATG1 and ATG13 [53]. On the one hand, TORC1 activity is critical for cell growth since TORC1 increases anabolism and decreases autophagic catabolism. On the other hand, when TORC1 activity is misregulated it can cause diverse pathologies associated with aging and obesity [50, 54].

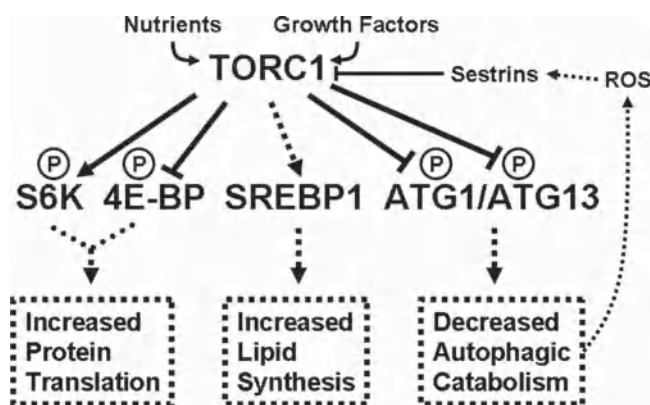


Fig. 24.2 Consequences of chronic TORC1 activation. TORC1, chronically activated by nutrient imbalance, can induce cellular anabolism of proteins and lipids while it can inhibit autophagic catabolism. Chronic TORC1 and diminished autophagy can cause accumulation of lipid droplets, protein aggregates, and damaged mitochondria, which can ultimately lead to diverse age-associated pathologies. Chronic TORC1-induced oxidative stress can induce Sestrin expression, which in turn acts as a negative feedback inhibitor of TORC1.

TORC1 is activated by two small GTPases, RAG and Rheb [51]. RAG mediates amino acid-dependent activation of TOR, while Rheb is negatively regulated by tuberous sclerosis complex 2 (TSC2), a GTPase activating protein. Lack of energy sources such as glucose and lipids that results in energy crisis causes activation of AMP-activated protein kinase (AMPK) [55]. AMPK activates TSC2 by direct phosphorylation, which subsequently silences the TORC1 activity by catalyzing hydrolysis of GTP bound to Rheb, a TORC1-activating small GTPase [56, 57]. Therefore, TORC1 is only active when nutrients are available. Conversely, diverse growth factors can increase TORC1 activity through AKT-mediated inactivation of TSC2 [47, 51].

24.6 CHRONIC TORC1 INDUCES STRESS-ASSOCIATED PATHOLOGIES

Chronic activation of TORC1 can cause various cellular stresses including endoplasmic reticulum (ER) stress, hypoxia, and oxidative stress [58–63]. The best-characterized function of TORC1 is its regulation of protein translation. The p70 S6 kinase (S6K) is activated by TORC1-dependent phosphorylation and subsequently phosphorylates ribosomal protein S6, which induces the translation of cell growth-related mRNAs [64, 65]. S6K also phosphorylates eukaryotic translation initiation factor 4B (eIF4B), which stimulates initiation of protein translation [66, 67]. At the same time, TORC1-dependent phosphorylation of 4E-BP releases eukaryotic translation initiation factor 4E (eIF4E) from 4E-BP-mediated inhibition [68]. In combination, these events cause increased protein synthesis, resulting in accumulation of unfolded proteins in ER and induction of unfolded protein responses [60]. Subsequently, cells having undergone TORC1 hyperactivation are more vulnerable to ER stress-induced cell death [60, 69]. TORC1-dependent increased protein synthesis and energy expenditure also cause a local hypoxic response and changes in the metabolic transcriptional program inside cells [61].

In addition to unfolded protein and hypoxic stresses, chronic TORC1 can result in oxidative stresses. Chronic TORC1 alters mitochondrial protein synthesis, rendering mitochondria inefficient in oxidative respiration and leading to the generation and accumulation of ROS [70–73]. Conversely, persistent reduction in TORC1 activity increases the efficiency of oxidative phosphorylation, contributing to the expansion of chronological life span. In conjunction with controlling mitochondrial protein synthesis, TORC1 can directly affect the quality of mitochondrial function, through removal of damaged mitochondria by a mitochondria-specific form of autophagy (mitophagy [5]). TORC1

phosphorylates the autophagy-initiating protein kinase complex [74–77], composed of ATG1/ULK1, ATG13, and ATG17/FIP200. ATG1 is the catalytic subunit of the complex, which is inhibited by TORC1-mediated phosphorylation [78–83]. ATG1 activity is also critical for mitophagy [84]. Therefore, TORC1 hyperactivation can cause chronic downregulation of ATG1 activity, subsequently reducing mitophagy, which then leads to the accumulation of damaged, ROS-producing mitochondria. Conversely, inhibition of TORC1 by starvation or rapamycin treatment induces autophagy including mitophagy [85, 86] and improves mitochondrial quality and hence energy conservation [4].

Autophagic defects cause mitochondrial dysfunction and oxidative stress in diverse model organisms. Depletion of ATG1, as well as ATG6, ATG8, or ATG12, causes accumulation of dysfunctional mitochondria and oxidative stress in the yeast *Saccharomyces cerevisiae* [87]. Depletion of ATG5 or ATG7 in mouse cardiac cells, skeletal muscle, and insulin-producing pancreatic beta cells induces accumulation of damaged mitochondria, which ultimately leads to oxidative stress and then organ dysfunction [88–92]. In *Drosophila*, ATG1 ablation causes severe degeneration of cardiac and skeletal muscles, which is associated with accumulation of damaged mitochondria and oxidative stress [12]. The neurodegenerative phenotypes observed in mice with autophagy-deficient neurons [93, 94] may be also associated with mitochondrial dysfunction and oxidative stress commonly observed in brains of Alzheimer and Parkinson disease patients [95]. Such chronic TORC1 activation and reduced autophagy can result in degenerative phenotypes [4]. Indeed, obesity, which is associated with chronic TORC1 activation, is also associated with cardiac dysfunction and neurodegeneration [50, 96–99]. Conversely, inhibition of TORC1 by caloric restriction or rapamycin reduced the incidence and severity of cardiac and neuronal dysfunction upon aging and stresses in various model organisms [100–103].

24.7 SESTRIN AS A SUPPRESSOR OF TORC1

Sestrins were identified as an inhibitor of TORC1, which can suppress TORC1-dependent cell growth [10] and anabolic processes [12]. Upon induction, *Sesn1/2* completely blocked the TORC1-dependent phosphorylation of S6K and 4E-BP in cells and caused cell size reduction [10]. *Sesn1/2* is a part of large protein complex that contains AMPK and TSC2. In the complex, *Sesn1/2* induces activating phosphorylation of AMPK and potentiates AMPK-induced phosphorylation of TSC2, which in combination silence TORC1 activity [10]. *Sesn2*-mediated inhibition of TORC1 can also induce

autophagy [11]. Given that Sestrins are induced upon diverse stresses, including genotoxic and oxidative stresses as well as hypoxia, they may mediate the stress-dependent silencing of TORC1 activity, which eventuates in inhibiting cell growth and induction of autophagy under more extreme conditions [8, 12, 104].

There is ample documentation of the TORC1-suppressing role of Sestrins in diverse cell lines, as well as in animal models [6]. Both in cells and in *Drosophila* tissues, overexpression of Sestrins caused cell size reduction mediated via inhibition of TORC1 [10, 12]. Since Sestrin is induced upon chronic TORC1 activation, Sestrin acts as a feedback inhibitor of TORC1 signaling. Indeed, loss of Sestrins enhanced clonogenic growth of cancer cells [10] or hyperplastic growth of *Drosophila* wing tissue caused by TORC1 hyperactivation [12]. *Sesn2*-dependent TORC1 silencing was also observed in the mouse lung [105], while in the liver it mediated DNA damage-induced silencing of TORC1 [10]. Similarly, in fibroblasts, *Sesn3* mediated oxidative stress-induced silencing of TORC1 [106]. Collectively, Sestrins can mediate stress-induced silencing of TORC1, ultimately attenuating cell growth and funneling the saved energy into cellular repair and protecting cells from damage-induced apoptotic or necrotic cell death.

24.8 SESTRIN DEFICIENCY RESULTS IN AGE-ASSOCIATED PATHOLOGIES

Although TORC1 is a critical regulator of cell growth [47] and Sestrin potently inhibits TORC1 activity when overexpressed [10, 12], we were unable to detect any gross developmental defects in cell size and growth regulation in *Sestrin*-null mutant flies [12], implying that Sestrin-dependent control of TORC1 is not critical for normal development and cell growth under standard laboratory conditions. Therefore, the role of Sestrins may only be important in the context of homeostatic regulation rather than developmental cell growth, although there may be combinations of circumstances in nature in which developmental events are also dependent on Sestrin regulation. Supporting a primarily homeostatic role for Sestrins, expression of *Drosophila* Sestrin increases with maturation and aging [12].

Sestrin-null mutant adult flies showed significant elevation of triglyceride level in the fat body [12], which provides functions similar to the mammalian liver [107]. In the fat body, Sestrin deficiency reduced AMPK activity while increasing that of TORC1 [12]. Fat accumulation was found to be dependent on AMPK-TORC1 regulation, since pharmacological activation of AMPK or inhibition of TORC1 relieved the accumulation

of triglycerides. Increased lipogenic gene transcription, which is associated with TORC1-dependent triglyceride accumulation [108], was also observed in *Sestrin*-null animals.

More striking phenotypes were discovered when we analyzed the heart physiology of the *Sestrin*-null mutant flies. That the heart is exquisitely sensitive to Sestrin function is consistent with the stringent metabolic and energy requirements in this tissue. In accordance with aging, *Drosophila* hearts spontaneously develop cardiac arrhythmia [109–111], and this age-associated cardiac degeneration is facilitated by TORC1 activation and suppressed by TORC1 silencing [96, 108, 112, 113]. Hearts from *Sestrin*-null mutant flies showed cardiac hypertrophy, increased heart period, and arrhythmicity, most of which were suppressed by pharmacological reduction of TORC1 activation [12]. It is very likely that cardiac arrhythmicity is associated with oxidative stress, as administration of antioxidant vitamin E as well as expression of catalase ameliorated the arrhythmic phenotypes. The cardiac degeneration is not a simple consequence of fat accumulation, because heart-specific downregulation of Sestrin expression, which does not cause fat accumulation in the fat body, can also induce cardiac degeneration [12].

In addition to the cardiac degeneration, *Sestrin*-null mutant flies exhibited skeletal muscle degeneration [12], which is also associated with aging [114]. The degeneration is preceded by mitochondrial dysfunction and ROS accumulation and is attenuated by antioxidant feeding [12]. The degenerative muscle phenotype as well as mitochondrial dysfunction were rescued by administration of the TORC1 inhibitors AICAR and rapamycin. Therefore, the AMPK-TORC1-controlling function of Sestrin seems to be important for regulating redox homeostasis in skeletal muscles. Furthermore, human Sestrin 1 is most highly expressed in skeletal muscle like its *Drosophila* counterpart [13], suggesting that Sestrin's role in muscle physiology may be conserved in mammals, which again highlights the importance of Sestrins in tissues with a high metabolic demand.

24.9 CONCLUSION AND PERSPECTIVES

As outlined in this review, biochemical and physiological functions of Sestrins revealed by mammalian cell culture and *Drosophila* genetics suggest that Sestrins protect cells and organisms against oxidative stress and age-associated pathologies as a feedback regulator. Upon acute oxidative stress, Sestrins function as a direct redox regulator to ensure cell survival. During chronic oxidative stress caused by TORC1 hyperactivation, Sestrins function as feedback inhibitors of TORC1,

which reduces TORC1-induced cellular anabolism and oxidative stress. Thus it is very likely that Sestrin homologs in mammalian organisms can also exert a protective function against oxidative stress and aging. Understanding the precise role of Sestrins in diverse pathological contexts may provide a novel way to attenuate metabolic derangement and tissue injury that are caused by oxidative stresses. Diversification of the Sestrin gene family in the mammalian genome suggests that each of them may have unique specialized functions. Although the biochemical and cell biological roles of Sestrins are indistinguishable between Sestrin members, the detailed mechanism for transcriptional regulation varies. Therefore, there is an obvious need for continued rigorous study of the specific roles of each mammalian Sestrin homolog in various tissues and physiological contexts.

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CURRENT ADVANCES IN THE STUDIES OF OXIDATIVE STRESS AND AGE-RELATED MEMORY IMPAIRMENT IN *C. ELEGANS*

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25.1 INTRODUCTION

Memory impairment is one of the diverse manifestations of the aging process. It can occur either as a part of normal aging or in association with a pathologic process. Age-related memory impairment (AMI) is a normal result of aging, which is observed in a wide variety of species from the nematode *Caenorhabditis elegans* to human. Recent studies suggest that aging causes heterogeneous effects: Some types of learning and memory are decreased, but other types are increased or show no change. AMI is not a gradual decay but rather an aspect of age-related alterations in the nervous functions. Recently, the studies of AMI in *C. elegans* have been emerging. However, there are misconceptions that remain to be clarified. The mechanism of AMI is thought to be related, at least in part, to oxidative stress, which is caused by an imbalance between the generation and removal of reactive oxygen species (ROS) and reactive nitrogen oxygen species (RNOS). Oxidative stress damages intracellular and extracellular components, including proteins, lipids, and DNA, and therefore it is predicted that aging and oxidative stress interfere with normal function of learning and memory. Detailing the mechanism of AMI will allow development of methods for identifying, delaying, and preventing both AMI and disease-associated memory impairment. It requires rigorous analysis of both aging neurons and the aging processes that affect learning and memory.

This chapter describes the emerging field of cognitive aging and discusses current understanding of oxidative stress and AMI, while referencing the insights from mammalian systems.

25.2 MEMORY IMPAIRMENT DURING AGING

Age-related changes in learning and memory can be seen as a form of “forgetfulness” in our daily life. This section provides basic information about AMI that is applicable from *C. elegans* to humans. We define terms and clarify major misconceptions.

25.2.1 Terminology

Age-related memory impairment (AMI), also called age-associated memory impairment (AAMI), includes mild impairment of the ability to learn new information and to recall previously learned information. The term age-associated cognitive impairment (AACI), is also used to describe memory impairment during aging. Similar to humans, we define AMI (or AAMI) as memory impairment in comparison with young normal controls and AACI as in comparison with age-matched normal counterparts. In this chapter, we use AMI but not AAMI, since AAMI is often confused with the transition state, MCI.

There is also confusion about the definition of short-term memory (SST), long-term memory (LTM), and

working memory. The confusion is mainly due to various definitions from different investigators [1]. In this chapter, we define the terms as follows: Short-term memory (SST) is a transient memory, which lasts for a short time (ranging from seconds to hours) and decays rapidly; long-term memory (LTM) lasts days to decades. How long do SST and LTM last in *C. elegans*? In a typical associative learning assay, SST typically lasts for several hours. LTM lasts more than a day in *C. elegans*. Additionally, SST is sensitive to cold shock and other conditions. More details are described in Section 25.3.2, “Associative Learning and Memory.” Importantly, SST and working memory are not totally different [1]. Working memory uses SST as a buffer of memory for maintaining and manipulating memory or for using to affect behavioral outputs [1, 2]. Typically, working memory uses multiple SST buffers. Working memory is more complex than SST as working memory assumes more functions. The term working memory was originally described in Miller *et al.* (1960) [3]. An early model of working memory has been developed in order to describe STM more accurately [4] than the classical modal model [5].

Memory can be classified into two classical forms, including explicit memory (or declarative memory) and implicit memory (or nondeclarative memory) [summarized in Ref. 2]. When you can determine whether you remember or not, memory is called “explicit” (you know that you know) [2]. Explicit memory includes memory for event (episodic memory) and memory for fact (semantic memory). In contrast, memory other than explicit memory is generally classified as implicit memory. Implicit memory includes memory for skills, habits, and behaviors (procedural memory) and classical conditioning. Classical conditioning associates a stimulus to predict important events, and therefore it is a form of associative learning and memory. Recent studies suggest that associative learning and memory are vulnerable to aging from *C. elegans* to humans (see below).

25.2.2 Age-Related Memory Impairment

AMI creates an increasing number of concerns in elderly patients [6, 7]. AMI is the first step toward mild cognitive impairment (MCI; transition state) and dementia (disease state) [8]. Alzheimer disease (AD) is the major type of dementia. Progression to dementia occurs at a high rate in patients with AMI (42% within 3 years) [9]. In humans, it appears likely that memory impairment occurs in the order of:

AMI → MCI → Dementia

The current understanding of AMI is that a wide variety of associative learning and memory are altered (impaired or increased) during aging from *C. elegans* to

humans [7, 10–14] (also see Section 25.3.2). Aging selectively affects working memory and some tasks of short-term memory and long-term memory [10, 13, and this Chapter]. It seems to be generally agreed that implicit memory is reduced in learning new information and procedures (procedural learning) and remains intact in old memory and well-learned procedures (procedural memory for long term) during aging [2]. Other types of implicit memory, including classical conditioning, are differentially reduced in a wide variety of species, including *C. elegans* [reviewed in Ref. 13; also see this chapter], in fruit flies [15], in bees [16], in snails [17], in rodents [18–20] and in humans [21]. Similar to implicit memory, explicit memory shows a decline in learning new information but retains existing memory well. It appears that plasticity to new environments is reduced. Our current model is that aging causes a reduced ability to refresh the memory previously formed. More details of mammalian AMI are described elsewhere [2, 22]. Cognitive aging in *C. elegans* is described below.

A key question in cognitive aging is how aging affects cognitive functions. It has been suspected that aging does not occur at the same speed [2] (also see Section 25.7.1). Cognitive aging is presumably caused by age-related processes and by the results of aging (i.e., age-related changes) in neurons and by modifiers of learning and memory. The model systems, including *C. elegans*, are useful to reveal the mechanisms underlying cognitive aging. Excitingly, the study of cognitive aging has recently begun in *C. elegans*.

25.2.3 Common Misconceptions

In the field of AMI, it is useful to know misconceptions that remain to be or have been clarified. We list up some examples we have encountered through communication with the experts. First, AMI is not a simple decrease in learning and memory. Recent studies suggest that aging causes not only declines but also increases in learning and memory [e.g., Refs. 10, 23]. AMI is not a gradual decay but rather an aspect of age-related alterations in the nervous functions. A good example is seen in visual memory. Older patients require briefer stimulus time than younger patients in motion discrimination [23]. Aging may reduce the function of the interneurons, which is inhibitory to motion discrimination, and improve motion discrimination in senescent neurons [23]. In humans, some but not all forms of short-term memory and procedural memory show age-related declines [10]. Therefore, it is essential to describe which type of learning and memory is altered and how it is altered.

Secondly, AMI is not a disease. AMI is a normal state prior to the disease state. What distinguishes AMI from

dementia besides cognitive deficits? A few lines of evidence from functional magnetic resonance imaging (fMRI) analysis suggest that reduced metabolic activity may be a hallmark of AMI in two hippocampal subregions (the subiculum and the dentate gyrus) [24]. More detailed analysis of aging neurons and aging processes is essential for the understanding the mechanism, prevention, and treatment of AMI.

Finally, AMI cannot be independent of aging. There is an argument that aging should be separated from AMI. However, aging affects learning and memory, which results in AMI. More precisely, AMI is caused by age-related processes that affect learning and memory. The age-related processes should include age-related changes in neurons as well as elsewhere, including modifiers and pathways for learning and memory. In fact, a type of AMI can be suppressed by serotonin inhibitors [25], which are good examples of such modifiers. It is unlikely that aging neurons are the sole cause of AMI. Thus, focusing only on neurons would miss important aspects of the mechanisms for AMI. Although the study of aging neurons has been emphasized, it is equally essential to investigate the aging processes.

25.3 COGNITIVE AGING IN *C. ELEGANS*

C. elegans shows non-associative and associative learning and memory. Both forms of memory show alterations during aging. This section overviews the studies of aging of learning and memory that have been emerging recently.

25.3.1 Non-associative Learning and Sensory Functions

Several studies have investigated aging of learning and memory in *C. elegans*. Similar to mammals, not all functions of learning and memory decline with increasing age. A form of non-associative learning, habituation (i.e., a simple reduced response after a repeated stimulus) [26], increased in old animals, while recovery from habituation was slower in the old compared to the young [27]. Glenn *et al.* (2004) showed that chemotaxis, including attraction to benzaldehyde and avoidance of octanol, declines during aging [28]. However, it is not clear whether the declines were caused by reduced motor activity or by reduced sensory functions.

25.3.2 Associative Learning and Memory

C. elegans shows implicit memory that includes classical conditioning. By classical conditioning, non-associative can learn to associate food [unconditional stimulus(US)]

with a stimulus [conditional stimulus(CS)] and use the stimulus to look for food. The stimulus is associated with food (US), forming US-CS association. Thus it is a form of associative learning and memory. CS can be temperature or a chemical stimulus (i.e., smell and taste); US can be starvation (absence of food) or noxious stimulus (e.g., electric shock, heat, some chemicals and heavy metals). There are three types of associative learning behaviors known in *C. elegans*. They include thermotaxis, chemotaxis (with conditioning), and basal/enhanced slowing response.

25.3.3 Thermotaxis Learning and Memory

The study of thermotaxis has provided the first evidence for aging of associative learning and memory in *C. elegans* [11]. In thermotaxis, *C. elegans* can learn to associate a temperature with food (food-temperature association) [29, 30]. Food-temperature association leads to the tracking of the given temperature, using short-term memory (STM). It has been shown that peak performance of temperature-food association declines with increasing age [11]. It is likely that STM for temperature-food association is declined during aging, since old animals that have normal locomotion show impaired thermotaxis.

Thermotaxis can be assessed either in a single-animal assay (isothermal tracking) or in a thermotaxis population assay. The single-animal assay uses a thermal gradient as shown in Figure 25.1A. Worms can learn to move toward a given temperature (16°C) when conditioned with food (temperature-food association). When a worm is placed in a thermal gradient created on an assay plate, it moves toward the given temperature and tracks the isotherm. Thermotaxis can also be assessed with multiple worms. In this case, conditioned worms are placed on a thermal gradient plate. The fraction of worms in the area that covers the given temperature is assessed. In contrast, when worms are conditioned with no food (starvation), they avoid the temperature (temperature-starvation association). Worms conditioned with starvation are placed on thermal gradient near the area of the given temperature.

25.3.4 Chemotaxis/Olfactory Learning and Memory

Naive *C. elegans* moves to an attractant odor. When *C. elegans* is conditioned with the attractant, such as benzaldehyde, in the absence of food, it is no longer attracted by the odor and exhibits avoidance behaviors (starvation-odor association). The type of chemotaxis avoidance is a function of associative learning and is not related to exhaustion of sensory or motor functions [31]. STM and LTM can be assessed with a single

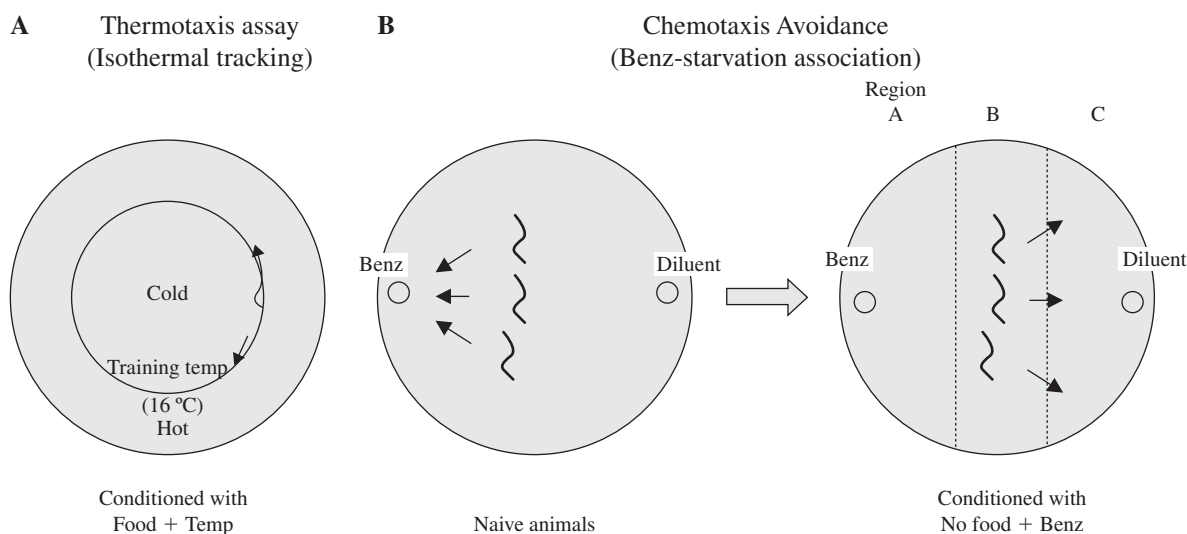


Fig. 25.1 Assay for learning and memory in *C. elegans*. **A**, Assay for thermotaxis learning and memory. The diagram represents an assay plate with a thermal gradient. A learning animal moves to and tracks the given temperature (16°C) created on the plate. **B**, Assay for chemotaxis learning and memory. Shown is a classical assay system for chemotaxis assay. Animals are placed in B and allowed to crawl. The chemotaxis index (CI) is calculated as follows:

$$CI = (A - C) / (A + B + C)$$

where A, B, and C are the numbers of the animals in each area. The learning index (LI) for avoidance assay is calculated as follows:

$$LI = (CI_{\text{naive}} - CI_{\text{conditioned}}) / CI_{\text{naive}}$$

When CI conditioned is less than 0, LI becomes more than 1. To avoid this overestimation, CI conditioned = 0 when CI conditioned has a negative value Benz, benzaldehyde.

conditioning and repeated conditionings, respectively. It has been shown that starvation-odor association declines during aging [32, 33] (Murakami *et al.*, unpublished). Although both STM and LTM decline, it appears that age-related decline in LTM occurs slightly earlier than that of STM (Murakami *et al.*, unpublished). Since LTM uses repeated conditioning with starvation, LTM may be more sensitive to age-related changes in starvation response than STM. Therefore, the results should carefully be validated.

There are several systems for chemosensory avoidance and attraction using different combinations of odor and taste [e.g., Refs. 11, 33–35]. Figure 25.1B describes a typical example of the assay system. Naive animals move to the attractant (benzaldehyde) (Figure 25.1B, left). When animals experience starvation with benzaldehyde, they avoid and move away from benzaldehyde (Figure 25.1B, right). The chemotaxis index (CI) and the learning index (LI) are defined in the Figure 25.1B legend.

25.3.5 Experience-Dependent Modulation of Locomotory Rate

The earliest change in associative learning behavior has been observed in another type of associative learning

and memory, called basal and enhanced slowing response [25]. Basal and enhanced slowing responses are experience-dependent locomotion behavior with associative learning properties [36]. When well-fed animals enter the presence of food, their movements slow to minimize the risk of moving away from the food area. This is called basal slowing response, and is regulated by dopamine signal [37]. Starved animals have a greater slowing response, called enhanced slowing response, than that of well-fed animals. This response is dependent on serotonergic signal [37, 38]. In normal aging, basal slowing response is increased, leading to a diminished difference between basal and slowing response [25]. It has been shown that the age-related change in slowing response is regulated by the serotonin pathway [25].

25.3.6 Neurons and Genes Relevant to Associative Learning and Memory

The neuronal circuits for thermotaxis associative learning consist of several types of neurons (Fig. 25.2). A pair of interneurons, AIY and AIZ, mediate thermal inputs from the thermosensory neuron AFD. These AIY and AIZ interneurons are required for associative learning; the thermosensory circuit shows conservation with the

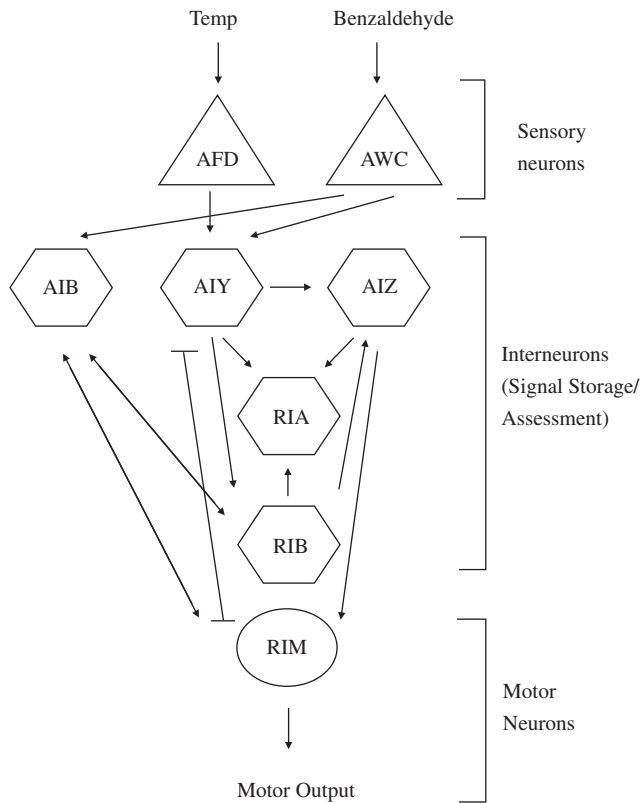


Fig. 25.2 Neurons relevant to thermotaxis learning and memory. Arrows, synapses; H-shaped bars, Gap junctions.

visual sensory circuit in vertebrates [39]. Thermotaxis learning requires the *ncs-1* neuronal calcium sensor gene [40] and the genes in the insulin/IGF-1 pathway (Fig. 25.3). A few modulators of thermotaxis learning are known. Serotonin mediates food responses associated with thermotaxis and olfactory associative learning [41, 42]. The *ncs-1* gene encodes an EF hand-containing calcium sensor protein, which is well conserved in a variety of species [40]. Expression of a neuronal calcium sensor protein gene, *ncs-1*, in AIY can increase isothermal tracking performance, while *ncs-1* knockout reduces the performance [40]. Another modulator, *hen-1*, encodes a secretory protein with an LDL receptor motif gene and is expressed in AIY and a chemosensory neuron ASE [43]. Mutations in *hen-1* abolish thermotaxis and chemotaxis learning behavior.

Olfactory learning requires sensory neurons for odors and some genes including *glr-1* (AMPA-like glutamate receptor gene). Benzaldehyde is primarily sensed by the AWC sensory neurons, which are chemosensory neurons for volatile odors (Fig. 25.2). Also of interest, mutations in *glr-1* impair olfactory associative learning when diacetyl is paired with acetic acid [44]; AMPA-type glutamate receptor mediates fast excitatory neurotransmission in the vertebrate brain. The *hen-1* LDL receptor gene is also involved in chemotaxis learning behavior when Cu^{2+} and diacetyl are paired [43]. There are four mutants that affect chemotaxis associative learning, including *lrn-1*, *lrn-2*,

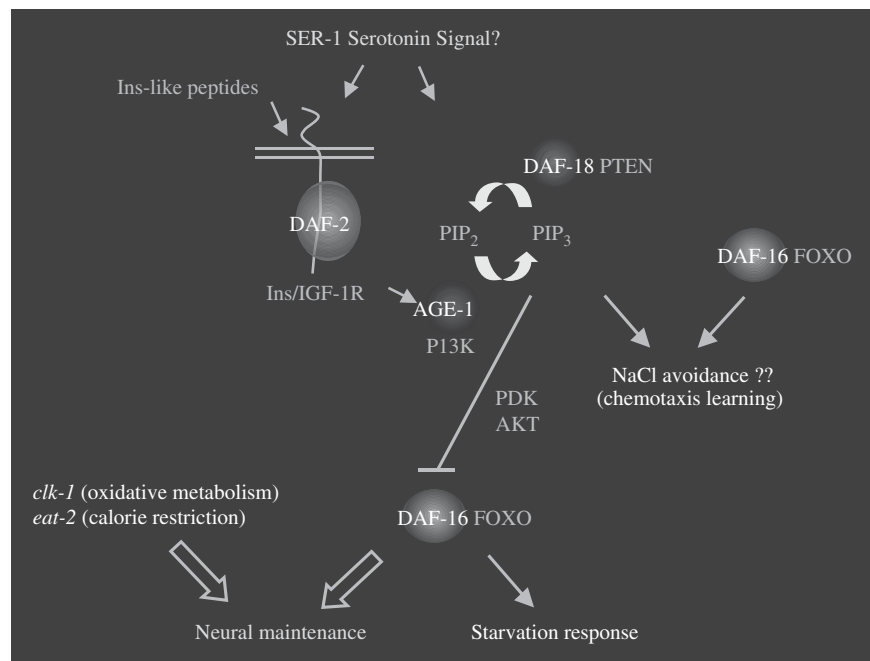


Fig. 25.3 A model for memory regulation by the insulin/IGF-1 pathway. It appears likely that the effects of life extension, or improved neuronal maintenance, lead to an increase in temperature-food association. (See color insert.)

JN603, and JN683 [34, 35]. Associative learning and memory are known to involve cAMP, calcium signaling and CREB in model systems including fruit flies, *Aplysia*, and rodents [45–47]. Locomotory associative-learning behaviors require dopaminergic neurons and serotonin-secreting neurons [37]. Basal slowing response uses the dopaminergic neurons, including CEP, ADE, and PDE, to sense mechanosensory stimuli. In contrast, enhanced slowing response uses serotonin-secreting neurons, including NSM.

25.3.7 *C. elegans* AD Models

The field is rapidly growing (196 publications by NCBI search, 2010) [reviewed in Refs. 48, 49]. Genes similar to those involved in human AD have been identified in *C. elegans*. They include App (*apl-1*), presenilin (*sel-12*), and Tau (*ptl-1*). *C. elegans* models of AD have been available as a form of transgenics expressing amyloid- β 1-42 in muscle [50]. Recently an improved version has been generated that express A- β in neurons [51]. Importantly, the life-extending mutants of the insulin/IGF-1 pathway can delay behavioral deficits in *C. elegans* and mouse models of AD [52, 53]. AD appears to be caused by proteotoxicity rather than aggregation of the proteins.

25.4 WHAT CAUSES AMI?

This section and the next section summarize age-related changes (and processes) that affect AMI. Important questions are how the changes are related to AMI and which changes are causes of AMI. This area of the study still remains to be explored rigorously.

25.4.1 Functional Alterations in Normal Aging

Age-related changes that affect AMI include (but are not limited to) increased oxidative stress, physiological alterations, neuroinflammation, reduced metabolic functions, and changes in endocrine function. Interestingly, microarray analysis has revealed two well-conserved processes, including reduced mitochondria metabolism (see Section 25.5.3, Reduced Mitochondrial Functions) and stress response in mice, monkeys and humans [reviewed in Ref. 54]. Stress response may be triggered by increased oxidative stress, which is consistent with the multiplex stress resistance model of aging [55]. Since microarray analysis determines relative abundance of mRNA, functional analysis should be performed to confirm the results. In addition, a large-scale functional analysis should be performed in order to overview the genetic basis of AMI.

25.4.2 Limited Neural Loss in Normal Aging

It was thought that cognitive aging is caused by a loss of neurons. However, it is becoming clear that a loss of neurons plays a relatively minor role. The number of principal neurons remains relatively intact during aging in *C. elegans*, rodents, and humans [56, 57]. *C. elegans* has a simple nervous system with a small number of neurons compared to other model animals [58], providing an ideal system to keep track of the fate of neurons. The structures of *C. elegans* neurons are relatively well preserved during aging, including the number of synapses [59].

In humans and monkeys, there is reduced brain volume that may be linked to cognitive aging [60, 61]. MRI studies suggest that healthy brains show a shrinkage in volume in humans [e.g., Ref. 61] and in monkeys [62, 63]. Neuronal loss plays a limited role in the shrinkage of the brain. The brain shrinkage is probably caused by shrinkage of neurons, reductions of synaptic spines, and lower numbers of synapses [61]. It seems consistent that brain shrinkage is observed in the region called white matter, which is rich in myelinated nerve fibers [64]. The integrity of myelin may be important for cognitive aging. The most affected areas in the brain include the frontal cortex and the temporal cortex. The frontal cortex is required for working memory, which is sensitive to aging. In contrast, the hippocampus remains relatively intact in the numbers of neurons and synapses. The hippocampus is critical for storing memory in classical conditioning; neural loss in the hippocampus is a warning sign of a disease state, including early AD [65]. Although the volumetric variable has been frequently linked to cognitive aging, there is little evidence for volume-cognition relations because of technical difficulties [22].

It is known that spatial memory can be impaired without neural loss [66]. In primates, neural loss occurs in the frontal cortex and in the cerebellum, including Purkinje neurons [67]. Purkinje neuron loss is well correlated with eye-blink conditioning deficits [66]. However, Purkinje neuron loss can be induced by various factors not necessarily specific to aging, including development, toxins, and autoimmune diseases [66, 68, 69]. Taken together, neural loss appears to play a limited role in AMI.

25.4.3 Oxidative Stress in Normal Aging

Oxidative stress occurs *in vivo* when ROS generated by normal metabolism are not fully scavenged. The free radical theory of aging, also called the oxidative stress theory of aging, assumes that there is an accumulation of oxidative damage in macromolecules during aging, which eventually results in dysfunction of biological functions [70, 71]. Although the role of oxidative stress

in life span specification has been challenged (see Section 25.8, Role of Oxidative Stress in Aging and AMI), it remains convincing that increased oxidative stress has an impact on age-related functional declines in biological processes. It has been shown that an indicator of oxidative damage on DNA [8-oxo-2-deoxynucleotide (oxo8dG)] increases with aging in rodent strains including F344 rats, B6D2F1 mice, and C57BL/6 mice [72]. Another indicator of oxidative damage, protein carbonyl, increases sharply in the last third of life span in a variety of species from *C. elegans* to humans [73, 74].

The central nervous system consumes a great deal of oxygen and is rich with components susceptible to oxidative stress, such as polyunsaturated fatty acids (PUFA, often referred to as “fish oil”) [75, 76]. In the rodent strains, brain is one of the tissues that show the greatest increases in oxo8dG levels (ranging from 167% to 340%) in 24-month-old compared to 6-month-old animals [72]. The increases in oxo8dG may be caused by increased sensitivity to oxidative stress.

In humans, gene expression is reduced in the frontal cortex, including the genes involved in synaptic plasticity, including NMDA and AMPA receptor function, calcium-mediated signal, and synaptic vesicle release and recycling [77]. The reduced expression occurs in the relatively early phase of normal aging (after the age of 40; mid-preproduction period).

25.4.4 Oxidative Stress in Neurodegenerative Diseases

There are extensive studies that suggest an association between increased oxidative stress and neurodegenerative diseases, including AD, Parkinson disease, and Huntington disease [e.g., Refs. 78–80]. Although the mechanism is to be determined, oxidative stress has been implied as a factor that promotes the initial phase of neurodegenerative diseases; other factors include glutamate signaling, altered calcium homeostasis, decreased growth factors, and genetic mutation [81]. For example, AD patients show increased levels of protein carbonyls, oxo8dG, and lipid peroxidation, especially in the brain area with amyloid plaques and neurofibrillary tangles [82]. In cultured neurons and in rat synapses, lipid peroxidation has been observed after exposure to amyloid- β , a component of amyloid plaques [e.g., Refs. 81, 83].

25.4.5 Mutations with Increased Oxidative Stress in *C. elegans*

Experimental evidence for the role of oxidative stress in AMI comes from a study using two mutational defects in the mitochondrial respiratory chain. The *mev-1* mutation has a defect in a cytochrome *b* large subunit (SDHC) in complex II in the mitochondrial electron

transport chain) [84]. The *gas-1* mutation has a defect in a subunit of the mitochondrial NADH:ubiquinone-oxidoreductase in complex I of the respiratory chain [85]. Both the *mev-1* SDHC mutation and the *gas-1* mutation cause overproduction of superoxide (O_2^-) and precocious aging [reviewed in Ref. 84].

It has been shown that thermotaxis is impaired by *mev-1* and *gas-1* mutations with increased oxidative stress [86]. Treatment of a natural pro-antioxidant, lipoic acid, can partially restore impaired thermotaxis in the *mev-1* mutant. It is plausible that oxidative stress plays a role in the performance of thermotaxis. Perhaps more convincing evidence for the role of oxidative stress has been provided by the studies of mutants and drugs that can reduce oxidative stress or oxidative metabolism (see Section 25.6.2).

25.5 OTHER FACTORS THAT MAY CAUSE AMI

This section continues on from Section 25.4, which describes potential causes of AMI. Listed below here are the factors that may affect AMI in *C. elegans* and mammals.

25.5.1 Physiological Alterations

AMI is in part caused by physiological changes in normal aging of the nervous system in mammals [7, 14]. It seems that AMI is caused by impairment of the ability to renew recent acquired information [87; this chapter]. Although neural loss is minimal, there is disruption of myelinated fibers, which may affect physiology of synapsis and communication among the brain regions in normal aging [88]. fMRI studies suggest that coordination between brain regions is significantly weakened in normal aging [88]. The elderly use a broader region of the brain (i.e., delocalization) than younger counterparts, which may be a compensatory response in the elderly.

25.5.2 Neuroinflammation

In normal aging, there is an imbalance between inflammation and antiinflammation, shifting toward a proinflammatory state. In humans and rodents, there is an increase in reactivity of microglia and astrocytes, which is indicative of increased innate immunity [89]. However, in humans, upregulation of genes involved in inflammatory is seen in the disease state, AD, while upregulation is weak in normal patients [90]. It is known that neuroinflammation is involved in an early phase toward AD and the diseases of aging [91] rather than AMI. It appears that patients with AMI are in a reactive but pre-inflammation state. Upon exposure to an inflammatory stimulus, including infection and stress, the

reactive state should trigger an overreaction of inflammation, leading to increased decrements in learning and memory [92]. Interestingly, anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), can reduce age-related cognitive decline in the elderly [93].

25.5.3 Reduced Mitochondrial Functions

The central nervous system relies on mitochondrial metabolism for energy production. Organismal aging in *C. elegans* shows reduced metabolic function [94], although energy production is uncoupled from life span extension in the *Clk* mutations that reduce oxidative metabolism [Ref. 94; also see Section 25.6.2, "Update"]. Microarray analysis has shown that mitochondrial genes show progressive declines during aging in humans, monkeys, and rats [reviewed in Ref. 95]. Importantly, proteomics analysis identified oxidatively modified proteins involved in energy metabolism and ATP production [96]. The modified proteins showed reduced activity. Oxidative stress also modifies the promoters of the mitochondrial genes [95], possibly leading to reduced expression of the genes. More detailed discussion about energy metabolism is available elsewhere [95].

25.6 IMPROVED LEARNING AND MEMORY

An exciting aspect for the studies of AMI is to identify genetic and pharmacological manipulations that rescue deficits in learning and memory. This section explores possibilities from previous findings.

25.6.1 Exploring Genetic and Pharmacological Interventions

Understanding of AMI will include neurological variables: (1) variables that cause AMI (e.g., modifiers) and (2) variables that are secondary or compensatory changes in response to the factors that causes AMI. The variables that cause AMI are reasonable targets for intervention, which is being explored. In *C. elegans*, such intervention includes mutations and drugs that reduce oxidative stress, alter neuroendocrine function (the insulin/IGF-1 pathway and the serotonin/octopamine pathway), and cause calorie restriction.

25.6.2 Mutants and Drugs that Can Reduce Oxidative Stress or Oxidative Metabolism

Mutants with deficits in mitochondrial transport chains are known to alter oxidative metabolism. The *isp-1* (iron sulfur protein gene) mutant decreases oxygen consumption

by half and therefore reduces oxidative metabolism [97]. Mutations in *clk-1* (mitochondrial di-iron carboxylase gene) lack an endogenous isoform of a redox-active lipid, coenzyme Q (Q) [98, 99]. The *clk-1* mutant accumulates a biosynthetic intermediate, demethoxy-Q₉ (DMQ₉) [99], that has a strong ROS scavenging activity. The mutants are *Clk* mutants and show increased longevity [100], stress resistance [101], delayed development, and extended rhythmic behavior cycles [97, 98].

A metabolic antioxidant, lipoic acid (LA), can improve the performance of thermotaxis [86]. LA is reduced to a potent antioxidant, dihydrolipoic acid, which can recycle other antioxidants such as vitamins C and E [103]. LA can decrease oxidative damage in the brains of older rats and partly restore age-related declines in nervous functions [104]. LA, along with statins (inhibitors of HMG-CoA reductase), can reduce LDL oxidation, which is also a risk factor of neurodegenerative disease and cardiovascular disease [105]. In mice, SOD/catalase mimetics (EUK-189 and EUK-207) can reduce oxidative damage (lipid peroxidation, nucleic acid oxidation, and ROS levels) and improve age-related decline in performance in the fear-conditioning task [106].

Update: During the review process of this chapter, a manuscript has been published that reverses the original claim that the *clk-1* and *isp-1* mutations reduce oxidative stress [97, 98, 100] to a new claim that the mutants modestly increase mitochondrial superoxide [102]. Importantly, the mutants show reduced mitochondrial functions, including oxidative metabolism, and, therefore, it is still reasonable to claim that the *clk-1* and *isp-1* mutations show reduced oxidative metabolism. Although the manuscript provides an interesting model that mitochondrial superoxide plays a role as a protective signal to mediate life extension [102], no data are presented to support the model, which remains to be tested.

25.6.3 Serotonin Pathway

Increased serotonin appears to alter behaviors during aging [25]. It is suggested that mRNA and expression of the serotonin biosynthesis gene, *tph-1* (tryptophan hydroxylase-1), are increased during aging [Ref. 25 and Murakami *et al.*, unpublished]. Consequently, reducing serotonin signal by serotonergic inhibitors and by the serotonin/octopamine pathway genes rescues age-related changes in basal/slowing response and chemotaxis avoidance [Refs. 25, 107, and Murakami *et al.*, unpublished]. Importantly, serotonin pathways also regulate life span [108].

25.6.4 Insulin/IGF-1 Pathway

It has been shown that the insulin/IGF-1 pathway modulates the late phase of age-related changes in

thermotaxis (temperature-food association) [11]. Figure 25.3 summarizes the current model; alternative models have been discussed elsewhere [13]. The insulin/IGF-1 pathway includes *daf-2* (insulin-like receptor gene) and *age-1* (phosphatidylinositol-3 OH kinase gene), which negatively regulate the Forkhead transcription factor, encoded by *daf-16* (Fig. 25.3). The *age-1* gene functions in the AIY, AIZ, or RIA interneurons [11, 109], suggesting that interneurons may be the core site of modulation by the insulin/IGF-1 pathway. Of note is the fact that proteotoxicity in the AD models is also suppressed by the mutations in the insulin/IGF-1 pathway (See Section 25.3.7, *C. elegans* AD Models).

Update: Several groups have confirmed that the insulin/IGF-1 pathway controls associative learning and memory in various *C. elegans* systems in young adults and during aging [33, 110–112].

25.6.5 Calorie Restriction

Interpretation of previous studies on calorie restriction (CR) requires careful consideration. CR can improve associative learning and memory in thermotaxis (food-temperature association) [86] and in chemotaxis avoidance (starvation-odor association) [33] in *C. elegans*. In contrast, CR in fruit flies does not: CR extends life span but does not suppress aging of an aversive learning task (odor-mechanical shock association) [113]. This may suggest a difference between the species. Alternatively, it may suggest a difference in experimental assay systems. The *C. elegans* studies use food (absence or presence of food) as a UC, while the fruit fly study uses mechanical shock as a UC. Thus an alternative model is that CR improves responses to food or starvation.

25.7 WHEN DOES COGNITIVE AGING BEGIN?

Although each form of learning and memory shows alterations at a different speed, current studies indicate two major time points that alter learning and memory in *C. elegans*. These include the early-mid reproduction period and around the end of the preproduction period, which appear consistent with human studies.

25.7.1 Multiple Phases of AMI

The studies of *C. elegans* suggest at least two phases of AMI. The early phase of AMI includes age-related decline in basal and slowing response and olfactory learning and memory (STM and LTM) [33, 86, 112]. The timing is at the early to mid-reproduction period. The late phase, in contrast, is around the end of the reproduction period. In the late phase, AMI includes disturbances

of various behaviors, including reduced thermotaxis associative learning behaviors and locomotion. It is unlikely that reduced locomotion at the late phase affects thermotaxis, as there is no correlation between reduced locomotion (by up to 40%) and thermotaxis in some mutations [86]. For example, the *clk-1* and *isp-1* mutations with reduced mitochondrial metabolism show reduced locomotion but an increased performance of thermotaxis [86].

Similarly, there are intriguing observations in humans. The cross-sectional studies suggest that some aspects of age-related cognitive decline begin in healthy, educated adults when they are in their 20s and 30s [114]. In contrast, the longitudinal data suggest around 60 years of age [115]. Although there seems to be a discrepancy, the two methods may be effective to reveal different phases of AMI in humans. It is notable that the standard cognitive tests use age-related adjustment, which artificially minimizes age-related changes [22]. The timing of the two AMI phases in humans is relatively similar to *C. elegans*. It is worth noting that there is an increase in oxidative damage around 40 years of age [77]. It is also possible that there are more than two phases of AMI. This line of experiments will be promising and should be performed for future studies.

25.8 ROLE OF OXIDATIVE STRESS IN AGING AND AMI: A THEORY

Oxidative stress increases with aging. However, the free radical theory of aging does not appear to explain aspects of aging. In this section, we propose a theory that incorporates evolutionary and biological aspects of aging.

25.8.1 Midlife Crisis Theory

Oxidative stress increases during aging and can damage macromolecules. The free radical theory of aging assumes that oxidative stress causes aging of biological functions [70]. Recent studies suggest that mutants in the superoxide dismutase (*sod*) genes show increased oxidative damage but fail to accelerate aging, providing evidence against the theory [116–118].

It is puzzling that ROS scavenging activities are relatively well retained, although oxidative damage increases during aging [73, 119]. In *C. elegans*, enzymatic activities of SOD (superoxide dismutase) and catalase do not decline until late in the life span (summarized in Fig. 25.4) [120]. This is consistent with mRNA levels of *sod* and *ctl* (catalase) genes, except for *sod-2*, declines with aging at mRNA levels [121].

Our midlife crisis theory assumes that oxidative stress is a part of an age-related crisis that occur in the middle

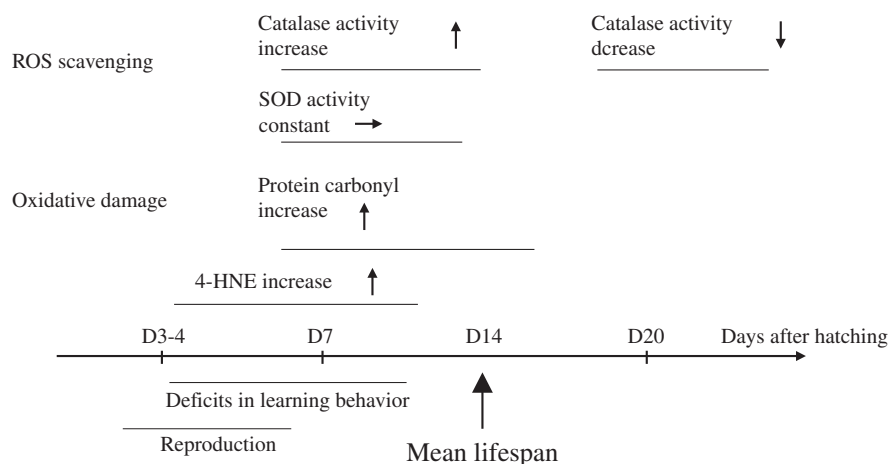


Fig. 25.4 A summary of *C. elegans* ROS and oxidative damage results during life span. Days are adjusted to the growth at 20 °C.

of the life span. It has been thought that normal metabolism in mitochondria is the primary source of oxidative stress [70]. Previous studies suggest that activities of mitochondria gradually change during aging [121]. If normal mitochondrial metabolism is the primary site of ROS generation during, ROS levels should gradually change during aging. However, an indicator of oxidative damage, protein carbonyl formation, increases in the middle of the life span [73]. The mechanism that causes age-related oxidative stress may be more complex than originally predicted by Harman (1956) [70].

The timing of increased oxidative stress is similar to that in which mortality increase, altered learning behaviors, and deteriorations of reproductive tissues are observed. We are inclined to propose a “midlife crisis” theory in which age-related increase in ROS is associated with the age-related deteriorations. In this theory, age-related ROS is associated with a wide variety of crises ranging from minor to major deficits. The midlife deficits occur around the end of reproduction, at which there is a diminishing force of natural selection. The deficits are unlikely to affect fitness of the species since they have a low impact on prosperity of the species. Therefore, the theory has an aspect of evolutionary and biological theory of aging. Importantly, in the theory, the *sod* mutations should have a limited impact on aging.

25.9 CONCLUSION AND PERSPECTIVE

AMI and neurodegenerative diseases impact more than 6.9 million people in the US [8, 123, 124]. Oxidative stress increases during aging and is a major risk factor for AMI and neurodegenerative diseases. Increased oxidative stress reduces expression of a specific set of neural genes during human brain aging [77] and is a

component of the transition state prior to the onset of neurodegenerative diseases such as AD [81]. It appears that aging neurons are sensitive to stress, including infections, oxidative stress, and environmental toxins. Such intrinsic and environmental stress may be partially rescued by nutritional approaches that include foods rich in antioxidants or anti-inflammatory compounds (e.g., berries, nuts, and grapes) [125, 126].

Despite the recent investigations, the genetic basis of AMI still remains to be determined. How does aging cause AMI? Can general aging be genetically delineated from tissue-specific aging that occur in neurons? Model systems, such as *C. elegans*, will provide useful systems to overview the mechanisms of AMI. Current understanding of AMI and oxidative stress appears to point out that oxidative stress and other age-related deteriorations occur in the mid to late phase of AMI. In the early phase of AMI, it seems that there is endocrine disturbance, as mutations in the insulin/IGF-1 pathway and serotonin pathway can rescue early markers of AMI. The emerging field of AMI is waiting to reveal its mechanisms.

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OXIDATIVE CHALLENGE AND REDOX SENSING IN MOLLUSKS: EFFECTS OF NATURAL AND ANTHROPIC STRESSORS

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26.1 INTRODUCTION

Mollusks are exposed to a variety of stresses in relation to their habitat and biology or to human activities. In aquatic environments, O₂ concentration and speed of diffusion are admittedly much lower than those in the air, but infested animals are likely exposed to an important range of oxygen concentrations with potentially great changes over time and space. Hence, coastal or estuarine animals can be subjected to tidal rhythm and consequently to oxygen deprivation during air exposure followed by reoxygenation at recovery. In tidal pools, and likewise in lake or lagoon shallow waters, animals are exposed to nyctemeral pO₂ variations due to O₂ consumption and production by algae. Pelagic mollusks are also likely to cross differently oxygenated water masses during vertical migration. Benthic mollusks live in sediments or at the interface with the surface, both characterized by low but variable pO₂. Concerning terrestrial snails that are air breathers, oxygen availability is quite constant unless the climate impels them to isolate from their surroundings to avoid desiccation or to limit gel formation. In this case, they undergo oxygen deprivation similarly to intertidal mollusks but for much longer periods, in addition to extreme body temperature.

All these situations induce oxidative challenges that mollusks are able to face under certain limits; indeed, they have evolved efficient defense strategies that allow them

to limit the harmful consequences of oxidative stress. Antioxidant defenses are close to those encountered in vertebrates, constituted by molecular and enzymatic actors and highly modulated as a function of environmental conditions. The antioxidant capacities and the relative contribution of the diverse antioxidant forces depends on the tissue, the species, or even the population for species living in heterogeneous habitats. For instance, high levels of antioxidant defenses are generally measured in animals living in permanent prooxidant conditions as in upper levels of the shore and in polar areas [1–3].

In addition to that ensuing from abiotic constraints, oxidative stress is also encountered in mollusks during microbial challenge, since their simple immunological responses consist in great part in triggering reactive oxygen species (ROS) surproduction by hemocytes to kill invaders with potential harmful side effects on host cells themselves. The last but not least aspect is the exposure of aquatic mollusks and, also terrestrial mollusks to a lesser extent, to environmental pollution due to industrial, agricultural, and domestic activities. A common denominator to a great part of these contaminants, notably organic compounds and metals, is the induction of oxidative stress due to direct toxicity or metabolism processes [4]. The discovery of the production of free radicals as a general feature of pollution exposure gave rise in the last two decades to a great interest in pro- and antioxidant processes in mollusks,

increasing the related knowledge for this phylum usually poorly studied. Hence, most of the data on antioxidant processes in mollusks were provided by ecotoxicological studies, in which antioxidants are used as biomarkers of chemical stress [4, 5].

The mechanisms of ROS formation, their deleterious effects on cellular components, as well as cellular defense responses are now better understood in mollusks and have been reviewed in several papers dealing with natural oxidative stress linked to ecology [6, 7] or with anthropic stresses [5, 8, 9]. Some papers only evoke the consequences of immune oxidative burst on the host itself [10, 11]. The cellular signaling processes involved in the response to variations in cellular redox status represent a field of research whose interest has grown recently. Related data in mollusks are provided by a few studies that address directly or indirectly this question by miscellaneous approaches. They allow us to understand some general features of these processes, but efforts in investigations have to be maintained to complete the picture. The aim of this review is to summarize current knowledge in the understanding of the cellular signaling response to redox variations in mollusks, in the frameworks of changes in O₂ availability and exposure to pollutants or pathogens.

26.2 OXIDATIVE STRESS LINKED TO VARIATIONS IN OXYGEN AVAILABILITY

Changes in O₂ environmental concentration are a major source of oxidative challenge in mollusks, mainly because of their original respiratory metabolism. In particular, intertidal and terrestrial mollusks are subjected to hypoxia-reoxygenation succession and exhibit adaptations that consist overall in regulation of energetic metabolism and redox balance to face this situation. These processes are triggered when tissular oxygen declines but part of them, for example, regulation of antioxidant forces, are involved above all in the cellular protection against the harmful consequences of recovery. Hence, mechanisms allowing the mollusk to face reoxygenation-induced oxidative stress encompass those underlying low O₂-sensing and downstream regulations, which are therefore described in this section.

26.2.1 Oxyconformity and Ectothermy

Mollusks are subjected to variations of abiotic factors that can change oxygen concentrations and consequently are likely to generate a recurrent situation of redox imbalance. The important influence of environmental oxygen on their oxidative status is linked to the fact that most mollusks are ectotherms and oxyconformers.

Ectothermy implies that body temperature follows that of the external medium. Oxidase functions and subsequent ROS production are a priori proportional to the temperature [12]. Hence, despite the decrease of O₂ solubility at high temperature, mollusks experiencing thermal stress likely suffer from oxidative stress all the more if the critical temperature inactivating antioxidant enzymes is reached [13]. On the other hand, oxidative stress can also be encountered in cold environments. In polar organisms, the lower metabolic activity and weaker speed of diffusion of oxygen in viscous fluids is counteracted by the fact that dissolved oxygen is more soluble and consequently more concentrated in seawater and body fluids of such animals than that of temperate animals [6]. Moreover, the adaptations of polar animals (homeoviscous adaptation, area of mitochondria) to facilitate O₂ transport and capture imply higher lipid content and higher proportion of unsaturation in membranes [6] so that equivalent levels of free radicals are actually registered and greater levels of oxidative damages are observed in certain species [14, 15]. In compensation, antioxidant capacities are generally more important in such organisms than in temperate organisms [2, 16].

In oxyconformers, oxygen consumption varies with environmental pO₂ but in a greater or lesser range according to their habitat and biology. Generally this conformity occurs above the low critical pO₂ until hyperoxic conditions, whereas in microoxic animals such as mud clams, oxygen uptake is independent from the environmental pO₂ above low concentrations [7]. At the molecular level, it has been shown that this conformity exists for both respiratory states 4 (unphosphorylating resting state) and 3 (phosphorylating active state) in mantle mitochondria of *Arctica islandica* and would be allowed by the alternative oxidase pathway rather than the futile cycling of protons (see next paragraph). Reduced aerobic activity when environmental oxygen is decreased may be necessary to avoid or at least delay tissular hypoxia and consequently limit the formation of ROS by reduced ubiquinone [7].

26.2.2 Hypoxia and ROS Production

Oxygen depletion in cell generates a slowdown of mitochondrial respiration and electron transfer, which leads to an increase in membrane potential, in reduction of complex III ubiquinone, and ultimately in ROS production, mainly superoxide anion [17, 18]. In mollusks, this production of ROS during hypoxia but also at recovery could be limited because of H⁺ leakage that can reduce membrane potential [7, 19]. This could be achieved by uncoupling proteins (UCPs) that evolved from the ancestral form of UCP2 and 3 known in

vertebrates, where they play among others a protective role against excessive ROS production [20]. In addition, it was shown that superoxide anions increase the expression of UCPs and activate them in rat muscle [21]. Another potential way to control ROS production when respiratory rate is low is the divergence of electron flow to an alternative oxidase (AOX) pathway by the influence of nitric oxide (NO). The latter interacts with cytochrome *c* oxidase of which it decreases the affinity for oxygen, leading to a shunt of electron flow into the AOX pathway [7]. Oxidases involved in the AOX pathway are expressed in diverse phyla of invertebrates as well as plants, fungi, and prokaryotes and can be transcriptionally induced by several kinds of stress (oxidative stress, hypothermia, pathogen infection, hypoxia) and their activity modulated at the posttranslational level [22, 23].

26.2.3 Hypoxia-Reoxygenation Challenge

Coastal as well as terrestrial mollusks are likely to encounter hypoxic stress in the way that the former undergo regular emersion and the latter isolate themselves from the surroundings when the aerial temperature reaches seasonal *extrema*, in order to limit water loss (aestivation) or gel formation (hibernation). In addition to the stress resulting from exposure to extreme temperatures [24], consequent deprivation of the medium of gas exchange implies that they are rapidly exposed to tissular hypoxia. To deal with these constraints that can occur frequently in intertidal species, anoxia-tolerant mollusks evolved adaptations of which the main adaptation is a metabolic depression up to 5% of the normal rate. This is possible because of a general breakdown of ATP-consuming processes and supply pathways in order that the ATP level remains constant [25–27]. Hence, the expression of almost all genes is suppressed, as well as translation, proteolysis, and part of the activity of ionic pumps with ATPase [28]. This situation already constitutes a challenge in itself, but the more harmful situation is the reoxygenation of tissues when animals are submerged or reawake. Indeed, when mollusks return to water, or emerge from lethargy in the case of land snails, the respiration rate increases suddenly and oxygen tension rises in tissues that were depleted and accumulated reducing equivalents. This situation is analogous to the ischemia/reperfusion phenomenon, which is known to lead to ROS overproduction and subsequent huge oxidative stress [29, 30]. Another situation comparable to ischemia/reperfusion is that generated by hyperthermia [7]. Indeed, rise in temperature of ambient water implies a parallel decrease of oxygen solubility as well as an enhanced metabolic activity. The combination of low pO_2 and increased O_2 consumption leads to a functional

hypoxia for animals exposed to such a stress. Thus, in addition to that ensuing from heat-induced ROS production, oxidative stress can occur when temperature declines and tissular pO_2 rises.

26.2.3.1 Triggering Hypoxic Response As in vertebrates, hypoxia triggers the activation of specific factors that regulate metabolism as well as the transcription of targeted genes whose products are involved in the cellular adaptation to low oxygen, but in a different manner to that of mammals. Few authors have taken interest in the understanding of low O_2 sensing in anoxia-tolerant mollusks, so that the mechanisms underlying these processes are still partially unclear, even if brand new findings help to complete the picture.

Low O_2 Sensing Most hypotheses concerning mechanisms of low O_2 sensing and regulation of gene expression are issued from mammal data that Larade and Storey reviewed in the context of anoxia-tolerant mollusks [26]. Hochachka proposed in the 1990s that O_2 itself could be a regulating molecule that directly controls the genic response to anoxia in anoxia-tolerant animals [28]. However, no evidence of the presence of a hypoxia-inducible transcription factor and associated regulators was provided in such organisms until recently [26]. Indeed, transcripts of hypoxia-inducible factor (HIF) α subunit as well as HIF-prolyl hydroxylase were newly isolated in *Crassostrea gigas* by Piontkivska and co-workers [31]. These transcripts share key functional domains and present a significant sequence similarity with invertebrate and vertebrate homolog. Exposure to 6-day anoxia did not significantly affect levels of these mRNAs, but they increased at recovery [32]. In fact, the α subunit of HIF-1 can be induced by hypoxia at the transcriptional level through a functional loop but the predominant regulation occurs at the posttranslational level [33], which may explain these results. Moreover, a 6-day air exposure is admittedly a challenge that oysters might encounter in wildlife, but it represents an extreme situation, and the consequent cellular response probably does not exactly reflect mechanisms involved in adaptation to cyclic tidal emersion. In any event, further experiments are required to understand the mode of action of this protein and the extent to which mechanisms are comparable with those of mammalian HIF-1 pathway. A priori, these findings allow us to think that in anoxia-tolerant mollusks like oysters, HIF- α would be oxidatively inactivated during normoxia by a prolyl hydroxylase, which needs oxygen to perform the HIF proline hydroxylation [33]. When oxygen tension declines, prolyl hydroxylases become unable to catalyze the oxidation of HIF- α , which can be translocated into the nucleus, interact with other subunits, and induce the transcription of hypoxia response element.

The nature of the hypoxia response element triggered by HIF in anoxia-tolerant mollusks is probably partly specific to these organisms, but the relative similarity in sequence and functional domains of oyster HIF- α with those of mammals suggests that some comparable genic responses such as NO synthase (NOS) expression could also be expected [7].

Second Messengers NOS activity has been detected in several groups of mollusks and has been particularly studied in the nervous system, where NO acts as a neuromodulator [34]. As an ancient and general biological regulator, NO is nonetheless involved in several functions of which are mediation of O₂ sensing and redox balance [34, 35]. NO is able to inhibit mitochondrial functions, and regulation of NO production is an important feature of hypoxic response [26, 35, 36]. However, there is a paradox in the fact that NO synthesis by NOS requires molecular oxygen and is consequently limited in severe hypoxia [35]. NO is a radical that is alternatively considered as a pro- or antioxidant according to the reaction in which it is involved and consequent products [38]. Hence, it can react with superoxide anion to form peroxynitrite (ONOO⁻), a highly cytotoxic radical, which is probably the major way of NO disappearance. This oxidation depends on O₂⁻ production and consequently on O₂ concentration. It has been shown that at low pO₂ NO half-life is longer than at physiological pO₂; hence, during hypoxia, NO degradation is lowered [36]. NO can also form nitrites NO₂⁻/NO₃⁻, which could constitute a form of storage of NO molecules. Mitochondria are sites of NO production, and this could be achieved by the reduction of nitrites when hypoxia occurs, probably thanks to cytochrome *c* oxidase [39, 40]. It is intriguing to note that cytochrome oxidase can both generate NO (at low pO₂) and have its oxidase activity inhibited by NO (particularly at phosphorylating and active state 3).

NO is also involved in hypoxia response through its role as activator of guanylyl cyclase and consequent influence on the production of cGMP [41], which most probably mediates O₂ sensing in anoxia-tolerant mollusks [26, 37]. Recent experiments suggest that guanylyl cyclases may actually not be activated by NO itself but rather by a complex of NO with glutathione, called *S*-nitrosoglutathione (GSNO). Indeed, it has been shown that GSNO, which has a hypoxia-mimetic activity, can activate guanylyl cyclase and that GSNO reductase, which reduces GSNO to restore reduced glutathione (GSH) and degrade NO in NH₄⁺ form, affects significantly NO-mediated hypoxic response [35]. Regulation of the GSNO pool could constitute a link between cellular redox status and signal transduction insofar as GSNO can generate nitrosothiols by transnitrosylation of protein sulfhydryl groups that may participate in cell

signaling. In addition, a relationship between the phosphorylation state of glycolytic enzymes and GSH content and synthesis was found in *Mytilus galloprovincialis* [42] that may be due to GSNO-induced cGMP production and subsequent activation of metabolic regulators. Indeed, the involvement of cGMP during hypoxia in mollusks seems to be linked to the cGMP-dependant protein kinase PKG, a key signaling protein that is known to mediate both phosphorylation of metabolic enzymes and anoxia genic response [26, 43]. Among numerous substrates, PKG activates the stress-activated kinase p38 MAPK that regulates the activity of transcription and translation factors, these functions being highly modulated in anoxia-tolerant animals [44]. In *Mytilus galloprovincialis*, p38 MAPK activation is apparently involved in response to different stresses (oxidative stress, osmotic stress, hyperthermia, exposure to diverse pollutants) [45–47]. Such stimuli also activate other stress-activated kinases (SAPK/JNK) at the same time as p38 MAPKs [47–48]; however, this is not the case for ischemia/reperfusion phenomenon in rat heart, where p38 MAPKs are activated during both anoxia and recovery but SAPKs only at reoxygenation [49]. This suggests that p38 MAPK could be a specific contributor to the response to anoxia-reoxygenation. Gaitanaki and co-workers found that anoxia exposure induced biphasic changes in phosphorylated rate of p38 MAPK in *M. galloprovincialis*: maximal values occurred at 1 h and 8 h of anoxia [45]. Interestingly, levels of phosphorylated p38 MAPK also increased transiently in the first 5 min of recovery after a 15-min air exposure. In *Littorina littorea*, an increase in phosphorylated p38 MAPK as well as phosphorylated 27-kDa stress protein (HSP27), which is regulated by the p38 MAPK downstream element MAPK-activated kinase 2 [51], was found in hepatopancreas after 12 h of aerial exposure [50]. HSP27 is a chaperone-like protein that increases cell resistance to heat shock or osmotic stress as well as short-term oxygen deprivation and myocardial ischemia-reperfusion, providing this protection in the latter situations by a role of the phosphorylated form in F-actin stabilization [52, 53]. Interestingly, 2-DE analysis on *Mytilus edulis* gills showed a downregulation of gelsolin, an efficient actin filament-severing factor, after 2 h of emersion on the shore [54], which is consistent with the idea of a reinforcement of the polymerization rate and stabilization of the cytoskeleton during hypoxic stress. However, activation of the p38 MAPK pathway is also involved in mediating apoptosis or necrosis during ischemia-reperfusion in myocardium and kidney [55, 56]; further experiments should help us to understand whether its role in the tolerance of mollusks to anoxia-reoxygenation is related to an enhanced cell resistance or an elimination of insulted cells.

26.2.3.2 Cellular Response to Oxygen Decline—Preparation for Oxidative Stress In addition to metabolic adjustments, signaling cascades and transcription factors activated by hypoxia most probably control the regulation of actors involved in the fight against oxidative stress. Evidence of such anticipated regulation is provided at the genomic level and through the modulation of antioxidant enzymatic activities during hypoxic stress.

Gene Transcription Contrary to mechanisms observed in anoxia-sensitive species, which consist mainly of compensatory mechanisms (expression of glycolytic enzymes and proteins involved in oxygen transport and vascularization), the genic response triggered in hypoxia-tolerant animals like certain mollusks generates products that are rather involved in altering metabolism, which allows them to withstand long-term oxygen deprivation [58]. Upregulation of certain transcripts has been shown in *Littorina littorea* during hypoxia, but most of them remain as latent mRNAs during this stressing period [37, 57]. These transcripts, which probably correspond to functions that are important for recovery, accumulate in the course of metabolic depression until normal conditions enable their translation. cDNA microarrays performed in hepatopancreas and foot of *L. littorea* allowed identification of a metallothionein [59] and a ferritin heavy chain [37] as upregulated genes. Both transcripts showed quick and important increase of expression and a maintenance of high levels throughout the hypoxia stress before declining at recovery. Resulting proteins are able to sequester metals and presumably participate in antioxidant defenses when free radical production is enhanced at recovery. Indeed, metallothioneins are shown to provide protection against oxidative stress in vertebrates [60, 61] and in invertebrates [62] due to metal binding but also to an inherent antioxidant function as ROS scavenger. Ferritin regulates Fe storage when its translation is not prevented by the binding to iron-regulatory proteins (IRP) that occurs when iron is low. This interaction is regulated by oxygen concentration [63]; in *L. littorea*, hypoxia probably triggers the dissociation of IRP-ferritin mRNA complexes, allowing ferritin translation. Management of the Fe pool and especially sequestration of free iron ions is a crucial aspect of coping with oxidative stress for mollusks [64]. In similar experiments in *C. gigas*, upregulation of glutathione peroxidase (GPx) and metallothionein transcripts were also observed in gills and mantle, which is consistent with the results described above [65]. At the proteic level, 2-DE experiments showed that a decrease of a thioredoxin peroxidase and Cu/Zn superoxide dimutase (Cu/Zn SOD) occurred between the end of emersion and 2 h of recovery in gills of *M. edulis* [54]. Both of these are antioxidants, and these decreases may indicate that they

are partially degraded after having contributed to the protection against oxidative stress at immediate recovery. Surprisingly, measurement of Cu/Zn SOD activity in the same study showed that it remained stable during hypoxia and increased after 2 h of recovery [54]. This discrepancy between expression and activity underlines that enzymatic antioxidant activities are regulated to a great extent at the posttranslational level. In this way, specific revelation of mussel Cu/Zn SOD activity after isoelectrofocusing reveals that the total activity visible on gel results from the contribution of three isoforms with different isoelectric points. The relative contribution of isoforms to total activity is different according to the tidal height of animals and reversibly modulated during the tidal cycle and by the pollution status of the environment [66, 67]. Authors suggest that these changes, which are clearly linked to cellular redox status, could correspond to interconversion of isoforms through oxidative modifications, perhaps as a functional response.

Regulation of Antioxidant Activities Earlier studies on the antioxidant response in mollusks exposed to air showed that antioxidant activities are modulated during hypoxia as well as at recovery according to species and tissue [6]. Most observations suggest that a spread strategy is an increase of certain antioxidant forces during hypoxia as a preparation to recovery, considering the probable adaptation to cyclic emersion or aestivation of anoxia-tolerant mollusks [68-70]. In *L. littorea*, antioxidant activities diminished during a 6-day exposure to N₂ and increased at recovery in hepatopancreas and foot muscle, but total glutathione increased during anoxia [69]. These changes in molecular antioxidant that are attributed to an anticipated adaptation to ROS generation seem efficient, since a decrease in lipid hydroperoxides was observed in hepatopancreas throughout the experiment. Almeida and co-workers exposed *Perna perna* mussels to air for 24 h and found that only glutathione transferase activity increased at the end of the anoxia period and remained high after recovery; on the other hand, increase in oxidative damages on lipids and DNA was reported during air exposure, with a return to basal values at recovery [29]. This suggests that oxidative stress occurs also during emersion for aquatic mollusks. In addition to the ROS generation by mitochondria due to electron flow lowering, another possible cause is gaping behavior. This consists in opening briefly the shell in order to extract aerial oxygen and increase pO₂ in water of the pallial cavity [71]. This behavior that significantly participates in energy production during emersion implies a sudden exposure to air of tissues that are oxygen depleted and consequently the generation of oxidative stress [29, 30]. In another experiment where mussels were exposed to air

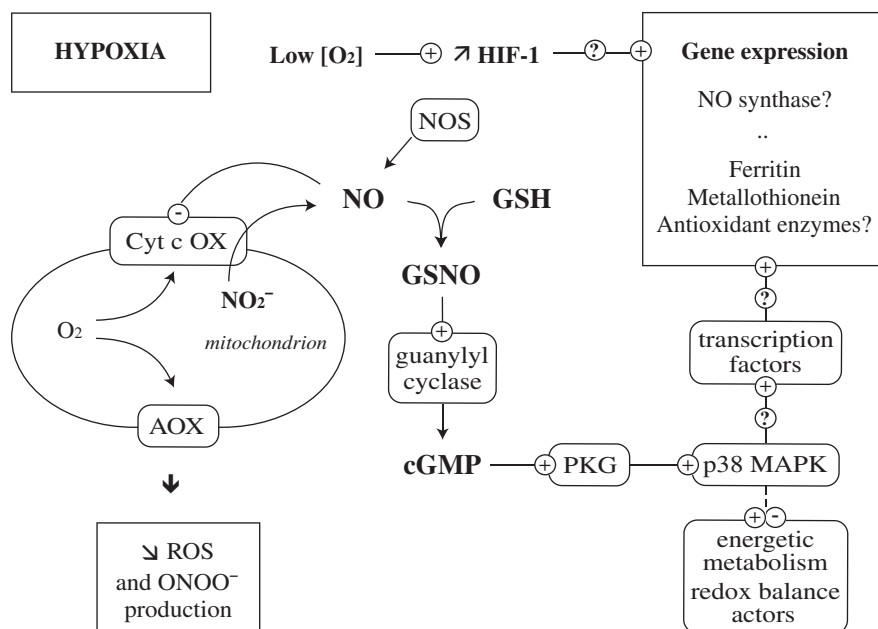


Fig. 26.1 A model describing modulation of mitochondrial functions and cellular signaling in response to hypoxia in anoxia-tolerant mollusks. Hypoxia triggers the production of NO, probably in part due to the mitochondrial reduction of nitrites. NO inhibits cytochrome *c* oxidase and causes a shunt of electron flow into an alternative oxidase pathway that allows minimization of ROS production. NO also associates with GSH to form *S*-nitrosoglutathione (GSNO), whose pool is regulated thanks to a GSNO reductase. GSNO complexes can then activate guanylyl cyclase to enhance cGMP production that probably helps to activate kinases like PKG. PKG in turn activates p38 MAPK, whose role could involve the activation or deactivation of metabolic and redox balancing actors. Also, it is possible that it initiates phosphorylation cascades until the induction of expression of targeted genes. In parallel, brand new information suggests that hypoxia genic response, at least in oysters, is triggered by the activation of HIF- α by a prolyl hydroxylase, but currently whether the mechanisms of transcription of responsive genes are comparable to those of mammals remains unknown. In this schematic hypothesis, signals triggered by anoxia generate a rapid metabolic response and a slower genic response that completes or reinforces the adjustment of energetic and redox processes.

for 4 h, which is more ecologically relevant, a short-term induction of SOD activity was observed in digestive gland at the end of emersion and considered as a preparation to reoxygenation [72]. In the pulmonate land snail *Helix aspersa*, a 20-day estivation led to an increase exclusively in GPx activity that the authors also attribute to a preparative mechanism, and 24-h recovery was characterized by a decrease in protein carbonylation and lipid peroxidation [73]. In an experiment where *M. edulis* was exposed to 6-h emersion, this strategy of anticipation appeared in gills, an interface tissue that is directly exposed to variations of environmental oxygen, but not in digestive gland, where levels of antioxidant activities seem to be dependant from metabolic state and consequently low at the end of emersion [54]. In field studies, tidal variations of antioxidant activities in *M. edulis* were admittedly significant but not drastic, suggesting that basal and sufficient levels of antioxidant capacities could be maintained despite the energetically unfavorable situation [54]. In addition, comparison of antioxidant status of mussels issued from low shore and high shore showed that individuals regularly exposed to emersion (high

shore) exhibit globally higher levels of antioxidant defenses than their subtidal counterparts [3], consistently with observations done on two close species of limpets living at different tidal heights [1]. Mussels that are regularly exposed to oxidative stress seem to be able to maintain constitutively high levels of defense as an acclimation, rather than adjusting them for any variation; such a strategy is also encountered in anoxia-tolerant turtles [74].

Even if gaps remain in our knowledge of cellular response to hypoxia in anoxia-tolerant mollusks and particularly of the involved transduction signals, the picture becomes clearer. Figure 26.1 presents a prospective scheme that summarizes the knowledge in this field.

26.3 POLLUTANT-INDUCED OXIDATIVE STRESS

Aquatic compartments constitute the ultimate receptacle of most environmental pollutants, so that organisms inhabiting such habitats are massively and chronically

exposed to a mixture of potentially toxic substances. Oxidative stress is a common consequence of xenobiotic presence responsible for biomolecule alteration. Some of these alterations constitute probable links with signaling processes that bring about cell death or, conversely, increase of resistance capacities.

26.3.1 Free Radical Production by Pollutants in Mollusks

As soft-bodied organisms, xenobiotics can enter in molluscan organisms through different ways (digestive tract by ingestion of water and food, respiratory surfaces, and across integumentary system) and accumulate because of low degradation capacities. Many compounds are implicated in the production of free radicals in mollusks, the main compounds being metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other organohalogenated molecules [38]. This toxicity is either due to the metabolization of xenobiotics in the case of organic molecules, although such capacities are limited in invertebrates [4], or the catalysis of redox reactions in the case of transition metals and depletion of thiol-containing antioxidants for other metals [75]. The ROS produced as a result of Haber–Weiss reactions or incomplete redox cycle, as well as organic peroxides that can be generated by cytochromes, can interact with biomolecules such as lipids, proteins, and DNA and, as in other organisms, generate damage that can be irreversible. Measurement of this damage (mainly products of lipid peroxidation and, to a lesser extent, DNA oxidation and fragmentation and protein damage) as well as induction of defense and repair mechanisms are used as biomarkers of oxidative insult in ecotoxicology [76]. Among the diverse oxidative attacks, protein and glutathione oxidations as well as DNA alteration probably play a role in the activation of pathways controlling cell response.

26.3.2 Oxidation of Proteins: Sensing Redox Changes?

Free radicals induce different kinds of oxidative modifications on proteins that can be reversible—and possibly involved in regulatory functions—or not [77]. A dramatic modification is the carbonylation of proteins, which consists of irreversible modifications of lateral chains into ketones or aldehydes and leads to protein aggregation and consequent inactivation and degradation. Measurement of carbonyl groups in soluble proteins is a tool used in ecotoxicology for evaluating oxidative damages to proteins induced by contamination [78]; new approaches now allow study of the specific patterns of proteins that are affected by these modifications [79]. In 2-DE experiments coupled with blotting

and specific immunorevelation of carbonylated groups, it was shown that levels of carbonylation increased in parallel to the intensity of oxidative stress in *M. edulis* and in *Ruditapes decussatus* but with tissue-specific and pollutant-specific patterns [80, 81]. In particular, these studies revealed that a major target of such oxidation in *M. edulis* is actin, which is known as one of the most sensitive cytoskeletal component to oxidant attacks [82]. Interestingly, revelation of ubiquitinated proteins in similar experiments done in *M. edulis* and *R. decussatus* showed that the print is different from that of carbonylated proteins, suggesting that the latter are degraded independently from ubiquitin-proteasome pathway [83–85]. Other modifications generally involve alteration of sulfhydryl groups, which are preferential targets of free radicals; oxidation of thiol functions gives rise to cleavage or formation of disulfide bonds that can be intra- or interproteins or occur between proteins and cysteines, glutathione, or even lipids. Disulfide bonds were thought to lead to the loss of protein function and inactivation of enzymes [86], but these bonds are reversible and could play regulatory roles in mild stress situations [87]. Glutathionylation of proteins, for instance, is a post-translational modification whose level increases in response to prooxidant treatment in diverse models. It allows the regulation of protein function and consequent modulation of cellular processes, protects cysteines during oxidative stress by preventing irreversible oxidation, and could play an essential role in diverse signaling pathways [88, 89]. It notably regulates the ubiquitin-conjugating activity and therefore controls the protease activity in mammalian cells [90]. An equivalent role of glutathionylation in the modulation of signal transduction can be expected in mollusks, but no evidence has been provided until now. Study of the glutathionylated proteome in gills and digestive gland of *M. edulis* showed that actin is again a preferential target of this modification [80]. This protein was slightly affected by inter- or intraprotein disulfide bond formation in redox proteomics experiments performed on tissues of mussels exposed to H_2O_2 [91]. A modified protocol helped to identify proteins touched by sulfhydryl oxidation in response to menadione treatment and revealed that proteic response to oxidative stress in *M. edulis* principally involves disulfide isomerases and cytoskeletal and chaperone proteins [92]. The role and mechanisms of these specific proteic modifications in cellular signaling and processes of mollusks have yet to be elucidated.

26.3.3 Pollutant-Induced Apoptosis

There is a correlation between apoptosis levels in mollusks and environmental stresses (salinity or temperature changes, pollution) whose common denominator is

oxidative stress. Stress-induced cell death, then, is probably linked to oxidative insult, and it is assumed that it is triggered by ROS excess and activation of the intrinsic pathway [93]. Genotoxic damages are a probable cause of the induction of cell death to prevent the threat of accumulation of DNA modifications. In mollusks, apoptosis is observed in response to chemicals in digestive gland, gills, mantle, as well as hemocytes and has to be distinguished for the latter from immune apoptosis occurring in case of pathogen attack. Exposure to sublethal concentrations of 4-nonylphenol gave rise to oxidative stress and hemocyte apoptosis in the clam *Tapes philippinarum*, with cell shrinkage and changes in morphology [94]. *Lymnaea stagnalis* exposed to a prooxidant pesticide showed an increase in hemocyte apoptosis with a time- and dose-dependent decrease in mitochondrial membrane potential [95]. Cd^{2+} induced hemocyte apoptosis in the oyster *Crassostrea virginica* in a dose-dependent manner with almost no effect at low concentration [96], and simultaneous exposure to elevated temperature significantly increased the occurrence of these events [97]. Curiously in oysters, Cd^{2+} -induced hemocyte apoptosis appears to involve a putatively original pathway different from the mitochondria/caspase pathway, since no decrease in mitochondrial membrane potential was recorded [96]. Cadmium introduced in the food also induced apoptosis in digestive gland of *Helix pomatia* [98]. The digestive gland is the major site of pollutant accumulation and metal detoxication; apoptosis probably allows the elimination of altered cells particularly in environments characterized by high heavy metal concentrations like hydrothermal vents, where such a phenomenon could be adaptative [99]. Apoptotic events are more rarely reported in gills, but DNA fragmentation followed by apoptosis has nevertheless been detected in gills of *M. galloprovincialis* exposed to single injections of different tri-*n*-butyltin (TBT) doses [100].

DNA fragmentation and changes in membrane potential strongly support the hypothesis of involvement of the intrinsic pathway in stress-induced molluscan apoptosis, but the exact mechanisms underlying this process are still obscure. In mammals, the key factor p53 is involved in both extrinsic and intrinsic apoptotic pathways. In the latter, redox changes activate p53, which promotes apoptosis either via nuclear translocation and gene transcription or by acting directly at the mitochondrial level, interacting with Bcl-2 proteins and inducing membrane permeabilization and release of cytochrome *c* [101]. In mollusks, there is evidence that equivalent pathways exist. Hence, human p53 homolog identified in leukemic *Mya arenaria* adductor muscle and hemocytes possesses highly conserved regions of functional domains, suggesting roles similar to those

of mammalian p53 [102]. Leukemic hemocytes of *M. arenaria* overexpress mortalin, a p53-binding stress protein that is responsible for p53 cytoplasmic sequestration [103]. Stress-induced DNA fragmentation led to the reversion of p53 cytoplasmic sequestration and translocation into the nucleus [103, 104], where p53 certainly promotes gene expression. The mechanisms of redox sensing by p53 are not clear, but depletion in cellular GSH is generally observed prior to the induction of apoptosis by p53 [105, 106]. Impaired glutathione redox status in mussel *M. galloprovincialis* and scallop *Flexopecten flexosus* has been associated with decrease in survival, which might be explained by GSH depletion-induced cell death [107]. Nitric oxide is also involved in apoptosis triggering, but its influence depends on dose, cell type, and cellular physiological status [108]. The exact role of these two compounds in apoptosis is not clear, but both are indirectly involved in the regulation of signaling cascades as a function of redox status, and in this view, activation of p53 may be attributed to upstream effectors such as stress kinases [109]. In addition, an increase in global tyrosine phosphorylation levels is observed in mussels exposed to various prooxidant chemicals (oil, mixture of oil/PAHs/alkylphenols, copper) [110]. In particular, p38 MAPK activation can promote cell death either through p53 pathway [111] or independently from this key regulator, possibly by direct activation of caspases [112, 113]. Involvement of p38 MAPK pathway in stress-induced apoptosis has been shown in *M. galloprovincialis* exposed to Zn^{2+} or Cu^{2+} since transient or biphasic activation p38 MAPK and increased levels of activated caspase 3 as well as DNA fragmentation have been observed in mantle [46].

26.3.4 Activation of MAPK Pathways in Prosurvival Response to Pollutants

In stress response, p38 MAPK regulates diverse pathways that can promote either cell death or resistance, depending on the cell type and the kinase isoform activated [109]. For instance, exposure to Zn^{2+} or Cu^{2+} induced in *M. galloprovincialis* gills a strong and long-lasting phosphorylation of p38 MAPK, while no DNA fragmentation or caspase 3 cleavages were detected at the same time, which contrasts with the results obtained in the mantle of the same species [46]. This suggests that activation of p38 MAPK by such a treatment in gills actually rather involves antiapoptotic signaling cascades, with upstream or downstream substrates able to reactivate p38 MAPK that would explain the persistence of phosphorylation [48]. Moreover, an increase in the levels of HSP70, a stress protein considered as an antiapoptotic factor [114], has also been registered in gills in

response to Cu^{2+} exposure [46]. Stress-induced HSP70 overexpression is generally observed at the same time as p38 MAPK activation in bivalves, probably because of induction of HSPs by MAPK prosurvival pathways in addition to protein damage, but it is notable that HSPs could themselves regulate signaling cascades [115].

Hence, tissues present a differential sensitivity to chemical stress, as suggested by the higher levels of chaperones in gills than in mantle [116], the diverse p38 MAPK pathways apparently activated by oxidant stimuli [46], and the differences in global phosphorylation levels observed in response to pollutant exposure [110]. This divergence of vulnerability could be due to a higher level of oxidative stress undergone by mantle cells, since mantle is more sensitive to lipid peroxidation [117] and gills possess higher levels of enzymatic antioxidant defenses [4]. The disparity in the response between tissues may also be due to a different “threshold” of stress level that activates proapoptotic pathways; indeed, it has been shown that MAPK activation involves different pathways and responses according to the severity of oxidative insult [118]. For instance, in human lymphoid cells, a high level of oxidative stress induced apoptosis, whereas

a low level induced mitotic arrest and probable transcription of antioxidant actors to balance redox status. Gills are the first tissue exposed to adverse factors, to which they probably exhibit greater tolerance than other tissues because of differential sensitivity and capacities to cope with a higher level of insult. Effects of diverse pr-oxidant contaminants on MAPKs in *M. galloprovincialis* gills were studied recently [47]. Low concentrations of TBT activated p38 MAPK and JNK but not ERK, and higher doses activated only p38 MAPK; in both cases DNA damages decreased, suggesting that the concentrations used in this study induced protective pathways of MAPKs, which contrasts with previous results obtained with very high doses of TBT directly introduced in the pallial cavity of mussels [100]. p38 MAPK/JNK activation and reduction of DNA damage were also observed in gills of mussels exposed to soluble fraction of diesel oil, indicating a probable role of MAPK activation in DNA repair [47].

Figure 26.2 proposes a schematic view of the hypothetical induction of apoptotic or resistance responses to oxidative stress in mollusks according to the severity of the insult, with p38 MAPK as a central regulator.

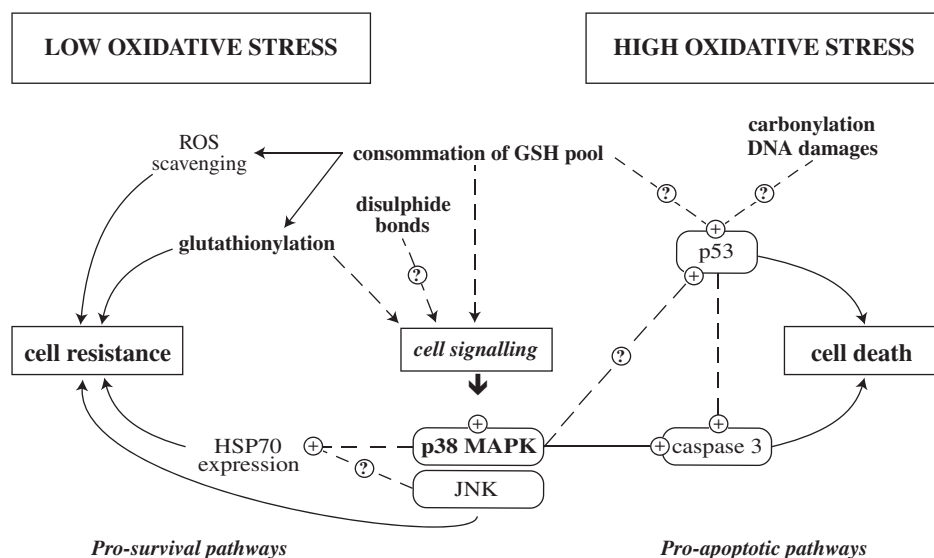


Fig. 26.2 A hypothetical model of the induction of apoptotic or resistance responses to oxidative stress according to the severity of insult. Oxidative stress induces the oxidation of thiol groups of proteins. Resulting disulfide bridges could play a role in signaling, while oxidation of free thiol is generally deleterious except in some cases (metal binding protein) where it allows scavenging of ROS. Glutathione is also oxidized or conjugates to proteins (glutathionylation), protecting them from irreversible modifications; in addition, glutathionylated proteins are also probably involved in signal transduction. Stress kinases, namely, p38 MAPK and to a lesser extent JNK, are activated probably in correlation with GSH levels. p38 MAPK induces the expression of HSP70 that protects proteins from damages and misfolding. These features allow enhancement of cell tolerance, which becomes capable of resisting oxidative stress if not too high. If oxidative stress is too severe, which is a relative notion depending on cell type sensitivity, p38 MAPK activates proapoptotic pathways by the cleavage of caspases, perhaps through p53 activation. Activation of p53 could also be regulated by GSH level more directly and by proteins or DNA damage. p38 MAPK plays a central role in the modulation of cell response to oxidative stress in mollusks.

26.4 IMMUNE SYSTEM

The molluscan immune system is less complex than that of mammals and involves humoral (lectins, hydrolytic enzymes, antimicrobial peptides) and cellular (hemocytes) mechanisms that recognize invaders and destroy them by various means [11]. Hemocytes are crucial components of this system and capable of migration, phagocytosis, aggregation, and secretion of antibacterial substances and ROS. One of the common features of molluscan host reaction against pathogen infection is the generation of an oxidative burst by hemocytes to kill microbes [119]. NADPH oxidase catalyzes the production of singlet oxygen, superoxide O_2^- , hydroxyl radical OH^\cdot , as well as hydrogen peroxide H_2O_2 that forms hypochlorous acid in a reaction with chloride catalyzed by myeloperoxidase, then enhancing antibacterial activity. It is nonetheless proposed that H_2O_2 could also originate from the Fenton reaction and hydroxyls result from interaction of H_2O_2 and superoxides. In addition, the amount of the different ROS produced depends on the cellular subtype as well as the stimulation [119, 120]. Oxidative burst is triggered by exposure to pathogens [119] but also carbohydrates [121], bacterial extracellular products [122], and cytokines [123]. Then, whereas it was thought that the phagocytosis itself activates a ROS-forming system associated with NADPH oxidase [119], it seems probable that ROS production is mediated by cGMP-dependent protein kinase PKC [124] and stress-activated MAPKs like p38 MAPK, as observed in *M. galloprovincialis* challenged with strains of *Vibrio* sp [120]. However, pathways triggered by a bacterial challenge depend on the pathogen as well as the extent and duration of the stimulation [125].

ROS production by hemocytes as a defensive reaction has the disadvantage of potentially inducing cytotoxicity to the host itself, which exhibits antioxidant forces to counteract this deleterious aspect of microbial killing. In addition to cellular antioxidants, circulating antioxidants are detected that could help to protect host cells from ROS insults. For instance, an extracellular superoxide dismutase has been characterized in oyster plasma [126]. This Cu/Zn form is exclusively expressed by hemocytes and is able to bind lipopolysaccharides (LPS) exhibited by bacteria such as *Escherichia coli* as well as integrin-like receptors on the surface of the oyster's hemocytes. In addition to putative roles in oxidative burst modulation and host protection against oxidative injury, this extracellular SOD could thus be involved in the recognition of LPS and subsequent triggering of the immune response by interacting with integrin receptors [126]. In the bay scallop *Argopecten irradians*, an extracellular SOD that does not possess

the LPS-binding motifs has been characterized, whose expression was enhanced in hemocytes when animals underwent *Vibrio anguillarum* challenge, probably as a consequence of ROS accumulation during oxidative burst [127].

If not balanced, ROS surproduced for bacterial killing may generate irreversible damage to host cells, even death. Apoptosis is observed as an important component of molluscan immune reaction to degrade infected or phagocytic cells [10, 128]. The mechanisms ruling this immunomodulatory process are still unclear, but oxidative burst probably participates in its activation, as observed for other environmental stress involving ROS formation [11, 95]. Hence, *C. gigas* hemocytes challenged in vitro with the Gram-positive marine bacterium *Planococcus citreus* phagocytized and killed invading cells within a few hours; then phagocytizing hemocytes, mainly hyalinocytes, underwent cell death that probably corresponds to apoptosis as suggested by preceding membrane blebbing, cell shrinkage, and chromatin condensation [11]. Besides, treatment with antioxidant agents suppressed phagocytizing-hyalinocyte death, whereas treatment of nonchallenged hemocytes with a prooxidant failed to induce apoptosis, suggesting that hyalinocyte apoptosis triggered by *P. citreus* exposure is actually induced by ROS produced within cells. Curiously, other experiments showed that granulocytes generally present higher levels of apoptosis than hyalinocytes, possibly due to higher phagocytic and respiratory burst activities [10].

In addition to ROS *sensu stricto* themselves, NO is also produced as a defense mechanism by molluscan hemocytes, where NO synthase activity has been detected, and different isoforms of NOS, some of them unique to mollusks, have been characterized [11]. NO synthesis occurs apparently after phagocytosis and would be involved in bacterial clumping [34, 123]. In the clam *R. decussatus*, NO synthesis seems to be independent from phagocytosis and constitutes an alternative method to kill pathogens [129]. In *C. virginica*, infection by the protozoan parasite *Perkinsus marinus* also induced NO production that was correlated with a decrease in parasite loads at early time points after infection [130]. In parallel, it has been shown that *P. marinus* increased hemocyte apoptosis at early and later stages of infection, with differential profiles according to the virulence of the strain [128]. Hence, the role of NO in the triggering of hemocyte apoptosis in mollusks is under question [10, 11]. Outside the immune system, the roles of NO in cell death are investigated in only one study that suggests an antiapoptotic role of NO in larval development [131]; on the other hand, data on mammalian macrophages show that stimulated NO production is associated with apoptosis of host cells [132, 133].

The putative role of ROS or NO in molluscan immune apoptosis intervenes certainly in addition to the direct stimulation of specific proapoptotic pathways by pathogen motifs and inflammatory or stress-induced endogenous molecules. In any case, the possible involvement of classical apoptotic actors like caspases is not elucidated, since divergent results are observed. In the abalone *Haliotis diversicolor*, bacterial challenge increased in a biphasic manner the expression of a specific caspase that is close to human caspases 8 and 10 [134]. On the other hand, recent work showed that *P. marinus*-induced apoptosis did not involve caspase activation in *C. virginica* [128]. Further experiments are required to understand the involvement of ROS and NO in immune apoptosis and the multiple pathways concerned with this process in mollusks.

26.5 CONCLUSION

Recent interest in signaling mechanisms involved in molluscan response to environmental stresses and study of oxidative modifications on the proteome allow us to gain insight into the mechanisms underlying redox sensing and cellular response to oxidative stress. Actors equivalent to those of mammals are generally concerned; in particular, MAPKs seem to play a key role and to control pathways and issues that vary according to the sensitivity of tissue or organisms and the severity of the insult. This provides interesting knowledge on molluscan stress response, which could be applied in the field of ecotoxicology and potentially used in biomonitoring studies. This also brings supplementary elements in the understanding of the mechanisms supporting anoxia tolerance in mollusks; however, a lot of information is lacking, and some considerations are speculative and require confirmation. Further investigations are necessary to elucidate remaining unknown mechanisms and give an integrated picture of the signaling processes involved in the anoxia-reoxygenation phenomenon. In addition, this review highlights the convergence of pathways involved in the response to diverse environmental stresses that could be undergone simultaneously by wild mollusks, therefore raising the potential interactive effects of multiple stresses.

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PERSPECTIVE AND DIRECTIONS FOR FUTURE STUDIES

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27.1 INTRODUCTION

Oxidative stress, an unavoidable consequence of life in an aerobic oxygen-enriched atmosphere, is a cytotoxic process that occurs in cells when antioxidant mechanisms are overwhelmed by reactive oxygen species (ROS). This imbalance causes damage to important biomolecules and cells, with potential impact on the whole organism [1]. ROS are atoms or molecules possessing one or more unpaired electrons in the outer orbit and, therefore, are prone to react chemically [2]. ROS include superoxide anions ($O_2^{\bullet-}$), hydroxyl ($\bullet OH$), alkoxyl (RO^{\bullet}), and peroxy radicals (ROO^{\bullet}), and hydrogen peroxide (H_2O_2). These radicals are common products of life in an aerobic environment, and they are responsible for oxygen toxicity. The “dark side” of oxygen is related to the fact that each oxygen atom has one unpaired electron in its outer valence shell, and molecular oxygen has two unpaired electrons. Thus atomic oxygen is a free radical, and molecular oxygen is a (free) biradical. Concerted tetravalent reduction of oxygen by the mitochondrial electron transport chain, to form water, is considered a relatively safe process; however, the univalent reduction of oxygen generates reactive intermediates. Thus the “oxygen paradox” is dangerous to all forms of life for whom it is an essential component of energy production [3]. The major sources of ROS include mitochondrial respiratory chain, xanthine/xanthine oxidase, myeloperoxidase in cytoplasm, uncontrolled

arachidonic acid (ARA) cascade, and NADPH oxidase (Fig. 27.1). Over 90% of ROS production occurs “accidentally” in mitochondria during metabolism of oxygen when some of electrons passing “down” the electron transport chain leak away from the main path and go directly to reduce oxygen molecules to the superoxide anion [4]. NADPH oxidase generates superoxide radical by the one-electron reduction of oxygen, using NADPH as the electron donor [5, 6]. The ability of NADPH oxidase inhibitors to retard ROS-mediated cytotoxicity provides strong support for the idea that ROS are generated through the activation of NADPH oxidase. In the presence of metal ions such as Fe^{2+} and Cu^{2+} , H_2O_2 is also transformed into hydroxyl radical ($\bullet OH$) through the Fenton reaction (Fig. 27.1). Hydroxyl radicals can attack polyunsaturated fatty acids in neural membrane phospholipids, forming the peroxy radical (ROO^{\bullet}), and then propagate the chain reaction of lipid peroxidation. ROS production plays an important role in cell signaling. ROS modulate the transcription factor NF- κ B through the activation of kinases that phosphorylate the inhibitory subunit of NF- κ B/I- κ B, causing its ubiquitination and release of NF- κ B from the NF- κ B complex. Free NF- κ B migrates from cytosol to the nucleus, where it binds to the κ B domain of the target gene promoter, leading to transcriptional activation of many proinflammatory enzymes, cytokines (TNF- α , IL-1 β , and IL-10), chemokines, immune receptors, and cell surface adhesion molecules (Fig. 27.1) [7], which are

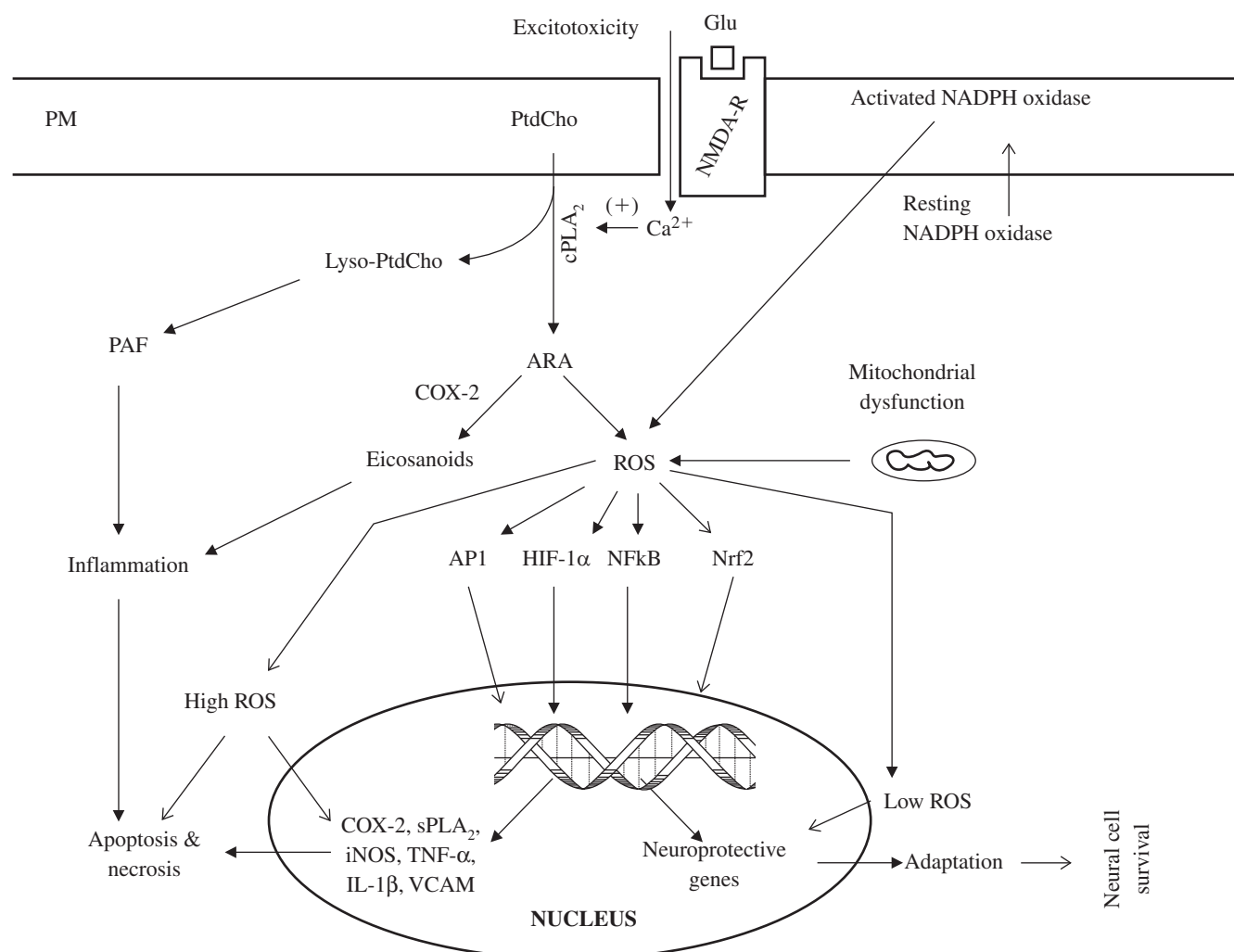


Fig. 27.1 Hypothetical model showing generation of reactive oxygen species (ROS) from various sources and effect of ROS on various transcription factors. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; sPLA₂, secretory phospholipase A₂; COX-2, cyclooxygenase; ARA, arachidonic acid; NF-κB, nuclear factor κB; iNOS, inducible nitric oxide synthase; PAF, platelet-activating factor; VCAM-1, vascular cell adhesion molecule-1; AP-1, activator protein 1; HIF-1α, hypoxia-inducible transcription factor-1α; Nrf2, NF-E2-related factor 2.

involved in different cellular processes such as cell proliferation, survival, stress responses, cellular immunity, and inflammation. Dysregulation of the NF-κB pathway leads to diseases such as autoimmunity and cancer.

Although chemical and biochemical reactions for the production of ROS in vertebrates and invertebrates systems are similar, cellular heterogeneity in various tissues and organs makes the intensity of oxidative stress responses very different. The consequences of oxidative stress depend not only on cell type and nature of ROS but also on endogenous antioxidant status and cooperation among various antioxidant systems [3]. In general, the preservation of redox status is a major factor for cell survival. Brains from vertebrate and invertebrate

phyla may respond differently in terms of intensity of oxidative stress compared to visceral organs of vertebrates (liver, kidneys, and lung) and similar systems in invertebrates. The mammalian brain demands high energy and possess highly active mitochondrial metabolism with high oxygen utilization (20% of the total oxygen inspired). This high utilization of oxygen comes at a heavy biological price. As important as oxygen is for the survival of neurons and glia, it also indirectly contributes to their destruction and death over time. The reason for this is that a small percentage (an estimated 1–4%) of the oxygen that enters cells is metabolized to derivatives that gradually erode and destroy essential molecules [8]. These destructive derivatives of oxygen are often referred to

as free radicals or ROS. Brain not only possesses high levels of polyunsaturated fatty acids but also contains transition metals such as iron and copper, which are capable of generating hydroxyl radical [9]. In addition, brain contains low levels of cytosolic antioxidants compared to liver and other visceral tissues [10, 11]. Thus brain is unable to protect itself from toxic effects of high levels of ROS. Superoxide radical reacts with nitric oxide (NO^\bullet) to produce the peroxynitrite anion (ONOO^-), a nonradical product, which is as toxic as $^\bullet\text{OH}$ in terms of its ability to oxidize and destroy by standard molecules. NO^\bullet and ONOO^- are often referred to as reactive nitrogen species (RNS). Like ROS, RNS oxidize lipids and protein components. Studies on ROS and RNS indicate that ROS/RNS are highly reactive and short-lived species that do not accumulate to significant levels, and it is not possible to measure them directly; rather, one must measure either the accumulation of biomolecules or the exogenously added indicators that are modified by ROS and RNS. In other words, generation of ROS and RNS leaves its footprint in the cell in the form of different oxidatively modified components.

27.2 ENDOGENOUS ANTIOXIDANT DEFENSE MECHANISMS IN VERTEBRATES AND INVERTEBRATES

Under physiological conditions, the antioxidant defense system within vertebrate and invertebrate bodies can easily neutralize the amount of ROS produced through ROS generating systems [2]. The antioxidant systems include low-molecular-weight antioxidants like glutathione and vitamin C, antioxidant enzymes such as superoxide dismutase, catalase, transferrin, and glutathione peroxidase, and the ROS defense system involving the participation of enzymes associated with DNA and membrane repair. These enzymes repair ROS-mediated oxidative damage to cellular structures [2]. Thus during normal aerobic metabolism ROS generation remains under tight control through the activities of the above-listed antioxidant defense systems. Low levels of ROS are needed for fundamental cellular functions such as growth and adaptation responses and for optimal functioning of the immune system. Oxidative stress induces a number of biochemical changes in vertebrate and invertebrate systems. The extent of these changes depends on the severity of the oxidative stress. In vertebrate and invertebrate systems, low levels of ROS promote cell proliferation, while intermediate ROS levels produce growth arrests [2, 8]. Under a sustained environmental stress, ROS are produced over a long time, and thus significant damage may occur to cell structure and functions. This damage has been implicated in a wide variety

of chronic diseases, including neurodegenerative diseases (Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis); arteriosclerosis and heart disease; strokes and ischemia/reperfusion injury; chronic inflammatory diseases (rheumatoid arthritis, lupus erythematosus, and psoriatic arthritis); and mutagenesis, cell transformation, and cancer [12, 13].

To counteract ROS-mediated damage, vertebrate and invertebrate systems have evolved several mechanisms. These mechanisms include (a) termination of the ROS using free radical scavengers and antioxidant enzymes, (b) induction of endogenous signaling systems that protect cells from toxic effects of ROS, and (c) repair of damaged cellular components. These mechanisms may also involve the activation of redox-sensitive transcription factors and increased expression of antioxidant enzymes and antiapoptotic proteins. One of the major cellular antioxidant responses in vertebrate and invertebrate systems is the induction of detoxification enzymes through the cytoplasmic oxidative stress system (Nrf2-Keap1). It is activated by ROS (Fig. 27.1). The transcription factor Nrf2 is constitutively expressed in all tissues of vertebrates and invertebrates, although amounts may vary among various cells and organs [14, 15]. The detoxification organs (kidney and liver) have the highest levels of Nrf2. Nrf2 may be further induced by cellular stressors including endogenous ROS or exogenous electrophiles [16]. Under normal conditions, Keap1 forms a complex with Nrf2 and keeps it within the cytoplasm, targeting it for ubiquitination and proteasomal degradation. This results in the maintenance of low levels of Nrf2 that mediate the constitutive expression of Nrf2 downstream genes. When vertebrate and invertebrate cells are exposed to oxidative stress, a signal involving phosphorylation and/or redox modification of critical cysteine residues in Keap1 inhibits the enzymic activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex, leading to reduction in Nrf2 ubiquitination and degradation. As a result, free Nrf2 translocates from the cytosol into nucleus, where it forms a heterodimer with members of the small musculo-aponeurotic fibrosarcoma (Maf) transcription factor family [15]. These Nrf2/Maf heterodimers bind to antioxidant response elements present in the promoters of numerous antioxidant genes, including NQO-1, glutathione *S*-transferase, glutathione peroxidase (GPx), catalase, and HO-1 [14, 17, 18]. Upon recovery of cellular redox homeostasis, Keap1 travels into the nucleus to dissociate Nrf2 from the ARE. Subsequently, the Nrf2-Keap1 complex is exported out of the nucleus by the nuclear export sequence (NES) in Keap1. Once in the cytoplasm, the Nrf2-Keap1 complex associates with the Cul3-Rbx1 core ubiquitin machinery, resulting in degradation of Nrf2 and termination of the Nrf2/ARE signaling

pathway [16, 19]. The Nrf2 signaling pathway mediates multiple avenues of cytoprotection by activating the transcription of more than 200 genes that are not only crucial in metabolism of drugs and toxins, protection against oxidative stress, and inflammation but also play an integral role in stability of proteins and in the removal of damaged proteins via proteasomal degradation or autophagy [20]. Nrf2 interacts with other important cell regulators such as tumor suppressor protein 53 (p53) and nuclear factor- κ B (NF- κ B) and through their combined interactions act as the guardian of a healthy life span, protecting against many age-related diseases such as cancer and neurodegeneration. It is hypothesized that this signaling pathway plays a critical role in the determination of species longevity and that this pathway may indeed be the master regulator of the aging process [21]. Since Nrf2-mediated cellular defense response protects multiple organs or multiple tissues, activation of Nrf2 has been implicated in conferring protection against many human diseases, including cancer, neurodegenerative diseases, cardiovascular diseases, acute and chronic lung injury, autoimmune diseases, and inflammation [18].

Oxidative stress also promotes both the transcriptional activity and protein stability of FoxOs, forkhead transcript factors that are expressed abundantly throughout the body of vertebrates and invertebrates [22–24]. FoxOs are major targets for insulin signaling. FoxO1 belongs to a nuclear protein subfamily that includes FoxO3a, FoxO4, and FoxO6 in mammals and its ortholog DAF-16 in *Caenorhabditis elegans* [25]. These proteins, characterized by a highly conserved central DNA binding domain and a carboxyl *trans*-activation domain, play important roles in mediating the effect of insulin or IGF on metabolism, growth, survival, differentiation of cells, oxidative stress response, DNA repair, cell cycle, and apoptosis [26]. Among the FoxO isoforms, FoxO1 preserves redox balance by promoting protein synthesis and subsequently inhibiting cell cycle arrest. This evidence indicates that FoxO1 integrates and orchestrates responses to different stress signals to maintain cellular function [22, 23, 26].

In addition, vitagenes play an important role in conferring protection against oxidative stress. These genes include genes for heat shock proteins (Hsps), thioredoxin/thioredoxin reductase system, and heme oxygenase-1 [27, 28]. Heat shock response contributes to establish a cytoprotective state in a wide variety of oxidative stress-mediated diseases, including inflammation, cancer, aging, and neurodegenerative disorders. When appropriately activated, heat shock response not only initiates and restores cellular homeostasis but rebalances redox equilibrium. Activation of this pathway is particularly important for neural cells with relatively weak endogenous antioxidant defenses [28, 29].

27.3 BIOMARKERS OF OXIDATIVE STRESS IN VERTEBRATES AND INVERTEBRATES

As stated above, direct measurement of ROS *in vivo* is difficult because of the highly reactive nature of these compounds and their minute concentrations in biological fluids. Instead, one relies on measurement of stable end products of oxidation of different molecules. ROS-mediated damage may occur to DNA, proteins, and lipids in vertebrate and invertebrate systems. Several markers of ROS-mediated damage have been described in the literature. An ideal biomarker for the detection of oxidative stress in chronic neurodegenerative and visceral diseases should be precise and reliable. It should be easy to quantify and be reproducible. It should not be subjected to wide variation in the general population and not affected by comorbid factors. To evaluate the effect of medication, the biomarker should change linearly with disease progression. Among biomarkers for oxidative stress, F₂-isoprostanes, 4-hydroxynonenal (4-HNE), and 8-hydroxy-2-deoxyguanosine have attracted considerable attention. F₂-isoprostanes and 4-HNE are compounds derived from arachidonic acid (ARA) via a free radical-catalyzed mechanism (Fig. 27.2). Several F₂-isoprostanes have been described in the vertebrate system, but it is becoming increasingly evident that 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) is a good biomarker for oxidative stress [30]. Isoprostanes are generated from cell membrane-bound ARA by free radical attack. They are cleaved from the sites of their origin by phospholipase A₂ and then circulate in plasma and are excreted in urine [31, 32]. F₂-isoprostanes can be detected in biological fluids, such as urine, blood plasma, bronchoalveolar lavage, or cerebrospinal fluid (CSF), as well as in tissues. The main advantage of urinary measurements of F₂-isoprostanes is that the compounds are very stable and are not formed *ex vivo* [33]. On the other hand, in blood plasma and tissues, autooxidation may occur. Several methods are available for F₂-isoprostane detection including gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry. In addition, a reliable ELISA technique suitable for analysis of large numbers of samples has recently been developed [32, 34, 35]. No information is available on the determination of isoprostanes in invertebrate systems. In future studies, attempts should be made to determine levels of isoprostanes in invertebrate systems by gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry.

4-HNE is the most prevalent toxic lipid peroxidation product formed during oxidative stress. It is derived from nonenzymic oxidation of ARA. It not only modifies amino acid residues of proteins (sulfhydryl groups of cysteine, imidazole moiety of histidine, and the ϵ -amino

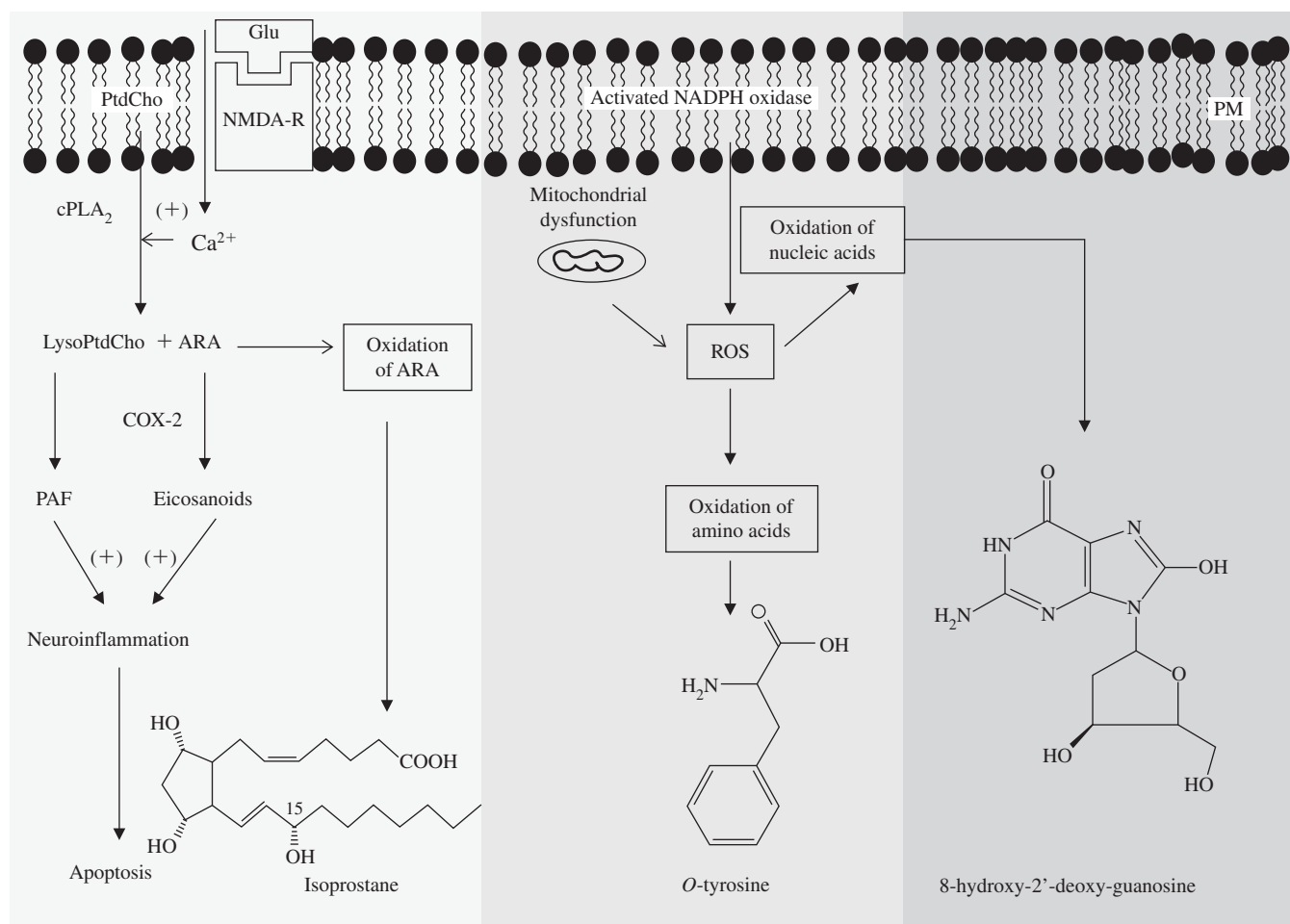


Fig. 27.2 Generation of biomarkers for oxidative stress in vertebrates. PM, plasma membrane; NMDA-R, *N*-methyl-D-aspartate receptor; Glu, glutamate; PtdCho, phosphatidylcholine; lyso-PtdCho, lyso-phosphatidylcholine; cPLA₂, cytosolic phospholipase A₂; COX-2, cyclooxygenase; ARA, arachidonic acid; PAF, platelet-activating factor; ROS, reactive oxygen species. (See color insert.)

group of lysine) but also inhibits DNA and protein synthesis and inactivates enzymes, modifies low-density lipoproteins, and modulates gene expression [36]. Mitochondrial proteins are targets of 4-HNE adduct formation following oxidative stress *in vivo* and *in vitro*. In addition, 4-HNE also inactivates the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, cytochrome *c* oxidase, and NADH-linked respiration in isolated mitochondria [37, 38]. 4-HNE produces a variety of cytotoxic effects such as the inhibition of DNA, RNA, and protein synthesis, cell cycle arrest, mitochondrial dysfunction, and apoptosis in vertebrate and invertebrate systems [39]. Thus 4-HNE is a good biomarker for oxidative stress. 4-HNE has been detected by gas chromatography-mass spectroscopy and liquid chromatography-mass spectroscopy in vertebrate systems, and studies on the use of 4-HNE as a biomarker for oxidative stress in invertebrates are beginning to appear [25].

8-Oxodeoxyguanosine (8-oxodG) is another important biomarker for oxidative stress in vertebrate and invertebrate systems [40–42]. It is synthesized *in vivo* by the attack of ROS on DNA. It is highly mutagenic, resulting in GC to TA transversions. After cleavage from DNA as a result of DNA repair, 8-oxodG is excreted in body fluids including blood, urine, and CSF of vertebrates. Another significant source of extracellular 8-oxodG may be oxidation of the nucleotide pool [43]. Urinary 8-oxodG levels are therefore considered a general biomarker of oxidative stress. Methods used for 8-oxodG detection include high-performance liquid chromatography, tandem mass spectroscopy, and a recently developed competitive ELISA [44]. These methods can be used for the determination of oxidative stress-mediated DNA damage in invertebrate systems.

Proteomics can be used for determining levels of not only oxidative/nitrosative protein modifications but also

protein-bound methionine oxidation [45, 46]. It is controversial whether ROS- and RNS-mediated protein modifications and oxidized methionines have a significant direct physiological and pathological impact on cellular injury. The generation of these proteins may be a secondary phenomenon. A clear delineation of the causal connections cannot be provided at present, but it is becoming increasingly evident that high levels of ROS/RNS produce distinct pathological cell consequences that greatly amplify and propagate injury, leading to irreversible cell and tissue degeneration [45, 46]. It is tempting to speculate that redox proteomics can be used to define redox molecular mechanisms associated with oxidative stress in vertebrate and invertebrate systems.

27.4 OXIDATIVE STRESS AND AGING

Although information on the effect of oxidative stress in vertebrate and invertebrate systems has been discussed by Rizvi and Pandey in this book, it is important to emphasize that production of ROS has a major impact on cell aging and tissue damage [47, 48], particularly in cardiovascular and nervous systems [49]. Levels of ROS are elevated with age, as biomarkers of lipid peroxidation (isoprostanes) are increased and antioxidant activity is decreased. However, in elderly individuals who are still healthy, the ROS level has been reported to be similar to that of young adults [50], or at least comparable to antioxidant defenses [51], supporting the view that markers of oxidative stress are not influenced by old age when good health and nutritional status are preserved. In addition, psychological stress and lifestyle factors such as smoking, lack of exercise, and status of n-3 fatty acids in the body have an impact on the level of ROS [52–54]. ROS are not only responsible for whole body accelerated aging but also for decline in cognitive functional. Thus in an elderly population (>80 years old), free radicals are involved in poorer cognitive function, loss of autonomy, loss of ability to perform daily activities, and institutionalization, as well as depressive symptoms [55].

27.5 CONCLUSION

Oxidative stress is defined as an imbalance between ROS production and their removal by antioxidant systems with increased accumulation of free radicals. Major production of ROS occurs in mitochondria during oxygen utilization. In addition, ROS may be synthesized in phagocytic cells, as well as in vascular wall and various other tissues by enzymes such as NADPH oxidase, myeloperoxidase, xanthine oxidase, cyclooxygenase, and lipoxygenase. At low concentrations, ROS are

associated with a vast array of physiological functions, such as gene expression and immune responses. At high levels, ROS react with lipids, carbohydrates, proteins, and DNA, altering their structure and function and resulting in inflammation, apoptosis, and mutagenesis.

These days, we have been empowered by lipidomics, proteomics, and genomics. These procedures can be used not only for identifying and determining levels of biomarkers for oxidative stress in various tissues of vertebrate animals and invertebrate organisms but also for detecting biomarkers levels in biological fluid samples. Identification of biomarkers for oxidative stress may lead not only to early diagnosis and follow-up of the progression of neurodegenerative and chronic visceral diseases but also to monitoring of therapeutic responses. Identification of ROS/RNS-modified proteins by proteomics in vertebrates and invertebrates can provide important information on cellular function of modified proteins. Another important challenge for future studies will be to incorporate this knowledge into a framework whereby these complex functional and regulatory alterations in the cellular proteome can be used to increase our understanding of and improve the treatment of diseases with the component of oxidative stress in vertebrate and invertebrate systems.

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